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CYTOCHEMISTRY AND MORPHOLOGY OF NEOPLASTIC AND NONNEOPLASTIC HUMAN LYMPH NODE CELLS WITH SPECIAL REFERENCE TO HODGKIN'S DISEASE [1] [2]

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The cytochemistry of normal and pathologic lymph nodes has not been explored extensively. In the lymph node of the rat a mucopolysaccharide (periodic acid-Schiff) has been described in the reticular fibers (*1*), connective tissue (*2*), some reticulum cells, and cells of lymphatic sinuses (*1*); and an acid polysaccharide is described in the intercellular substance (*2*). Lymphocytes in lymph nodes of the rat contain little or no demonstrable mucopolysaccharide (*1*, *2*). Gomori (*3*, *4*) was unable to detect acid phosphatase or lipase activity in human lymph nodes. Nachlas and Seligman (*5*) failed to demonstrate esterase in lymph nodes of man and of the dog, rabbit, rat, and guinea pig. Gomori (*6*) noted alkaline phosphatase activity in the peripheral zone of lymph nodules of certain species. This investigator found acid phosphatase and alkaline phosphatase in the spleen of the dog (*7*) and demonstrated various types of phosphatase in lymphatic nodules (*8*). Reticulum cells of the vermiform appendix of the rabbit show both acid and alkaline phosphatase activity; and phagocytic cells possess a high acid phosphatase activity (*9*). Endothelial cells lining the lymphatic sinuses of human lymph nodes exhibit no alkaline phosphatase, although those of the blood vessels show a high alkaline phosphatase activity (*10*). Some normal lymphocytes contain alkaline phosphatase (*10*, *11*); alkaline phosphatase was demonstrable in lymphocytes from 1 of 13 patients with chronic lymphatic leukemia (*11*), while those of acute lymphoid leukemia contained none (*10*).

The present paper represents a cytochemical study and a qualitative evaluation of the relative concentration of ribonucleoprotein, desoxyri-

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[3] The authors wish to acknowledge the technical assistance of Constance H. Eng and Beverly W. Mynchenberg.

[4] Deceased May 14, 1951.

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bonucleoprotein, mucopolysaccharide, hyaluronic acid, glycogen, lipid, lipase, and acid and alkaline phosphatase in the cells of human lymph nodes.

MATERIALS AND METHODS

Biopsy specimens of 102 human lymph nodes were studied by cytochemical techniques. For the purpose of description, the cells of the human lymph nodes will be placed in three general categories: 1) apparently normal cells of nonneoplastic lymph nodes; 2) modified cells of lymph nodes with lymphoblastoma involvement; and 3) modified cells of a group of miscellaneous lymph nodes. Table 1 summarizes the numerical distribution of the neoplastic and nonneoplastic human lymph nodes examined cytochemically.

TABLE 1.—*Neoplastic and nonneoplastic human lymph nodes examined cytochemically*

Diagnosis	Number of cases
Nonneoplastic:	
Lymphadenitis (chronic and subacute)	18
Reticulum-cell hyperplasia [1]	11
Lymphoid hyperplasia	3
Lymphoid hypoplasia	2
Follicular hyperplasia	2
Lymphoblastoma:	
Hodgkin's disease	24
Reticulum-cell sarcoma	5
Lymphosarcoma	7
Lymphatic leukemia	9
Acute monocytic leukemia	1
Giant follicular lymphoblastoma	4
Miscellaneous:	
Boeck's sarcoid	4
Infectious mononucleosis	1
Carcinoma:	
Breast	5
Bronchiogenic	3
Nephrogenic (?)	1
Unknown	2
	102

[1] Including 1 node obtained from a patient with congenital hemolytic icterus and 1 with thrombocytopenic purpura.

Representative portions of each lymph node were routinely fixed in Baker's formol-calcium, formol-Zenker, cold acetone, and Rossman's fluid. Tissues fixed in formol-calcium were embedded in Carbowax 1540; the other tissues were embedded in paraffin.

CYTOCHEMICAL TECHNIQUES

Lipid: A 70-percent alcoholic solution of Sudan black B (30 minutes) was employed for the demonstration of lipid (*12*). Neutral fat was demonstrated with a saturated aqueous solution of Nile blue sulfate according to the method outlined by Cowdry (*13*). Formol-calcium fixed sections were used in both techniques.

Nucleoprotein: The nucleal reaction (*13*) was used for the demonstration of desoxyribonucleoprotein. The material that gave a basophilic reaction following exposure to a dilute solution of toluidine blue (0.2 percent), and which could be digested out by prior incubation in ribonuclease (0.2 percent for 8 hours at 37° C.), was regarded as ribonucleoprotein. Tissues fixed in formol-Zenker have been used satisfactorily in our laboratory for nucleoprotein studies in spite of the unsuitability of dichromate fixatives reported by Stowell (*14*).

Enzymes: The acid and alkaline phosphatase reactions were carried out according to the procedure of Gomori (*15*). Ascorbic acid was added to the acid phosphatase substrate in order to augment the reaction (*16*). The lipase reaction (*17*) was employed in earlier experiments using Tween 40, 60, and Product 81. Lipase activity could not be demonstrated in lymph-node sections examined during this investigation. Acetone-fixed sections were incubated in the appropriate enzyme substrate for 18 hours.

Mucopolysaccharides, Glycogen, Sphingolipids (?) and Unsaturated Lipids (?): The periodic acid-Schiff (PAS) reaction was used for the demonstration of substances containing a 1,2-glycol linkage and may therefore be used to detect mucopolysaccharides (*18*), saliva labile glycogen (*18*), and perhaps sphingolipids (*19*) and unsaturated lipids (*20*). PAS-positive saliva-resistant material will be referred to in this text as PAS-positive material and usually will be considered as a mucopolysaccharide. Hyaluronidase was employed prior to the PAS and toluidine blue reactions in order to demonstrate the possible presence of hyaluronic acid; however, no difference could be detected in any of the cellular elements studied following this technique.

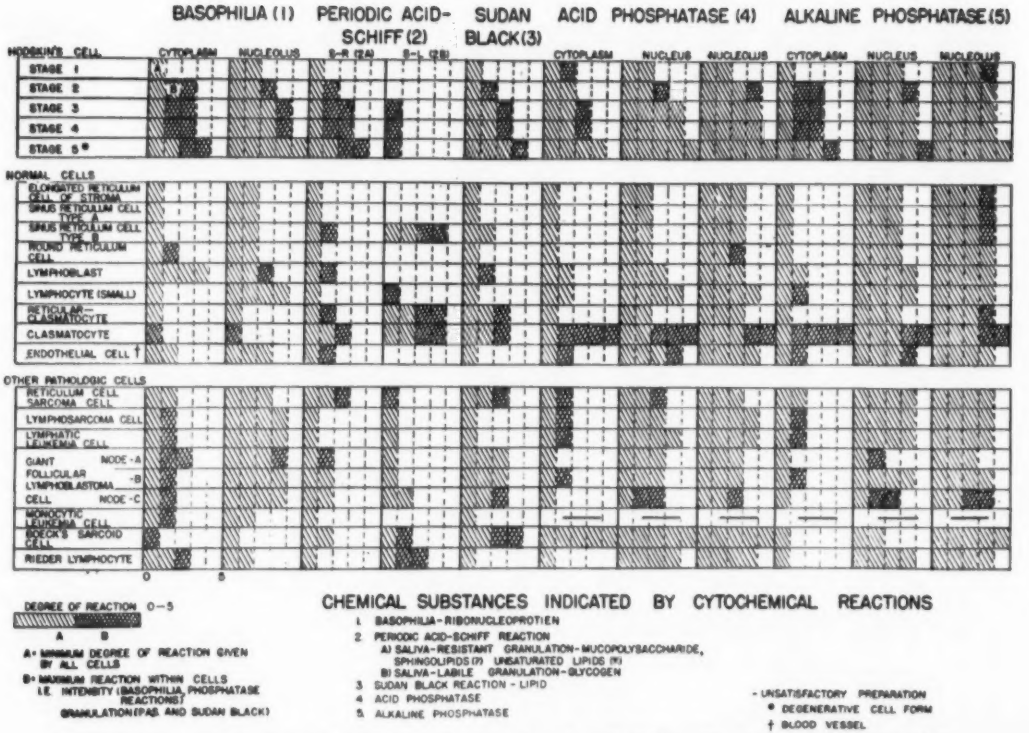
The cytochemical reactions that form the basis of this study have been evaluated on a numerical basis, the intensity of the reaction (toluidine blue and phosphatase), and the amount of granulation (PAS and Sudan black techniques), ranging from 0 to 5 (text-fig. 1).

OBSERVATIONS

Cells of Nonneoplastic Human Lymph Nodes

1) *Reticulum cells:* Reticulum cells of the normal lymph nodes may be placed in three general categories: (a) elongated cells comprising the stroma; (b) elongated cells forming the sinuses ("sinus endothelial cells"); and (c) round cells, observed in largest numbers in the secondary lymphatic nodules.

a) *Elongated reticulum cells of the stroma:* These are described in text-figure 1. The spindle-shaped reticulum cells comprising the stroma of the lymph node exhibit long, delicate, cytoplasmic processes, vesicular, centrally located nuclei, and one or two small nucleoli. The cytoplasmic ribonucleoprotein is colored light blue or lavender (+) with toluidine blue. Small periodic acid-Schiff (PAS)-positive and sudanophilic granules are located in the cytoplasm of these cells. Alkaline phosphatase activity is demonstrable in the cytoplasm (+), nucleus (+++) and nucleoli (+++-++++) (fig. 1). Acid phosphatase activity is greater in



TEXT-FIGURE 1.—Cytochemical reactions of normal and pathologic cells of human lymph nodes.

the nucleus (++) than in the cytoplasm (+) and the nucleolar acid-phosphatase content is usually greater (+++) than the acid-phosphatase content of the nucleus (fig. 2).

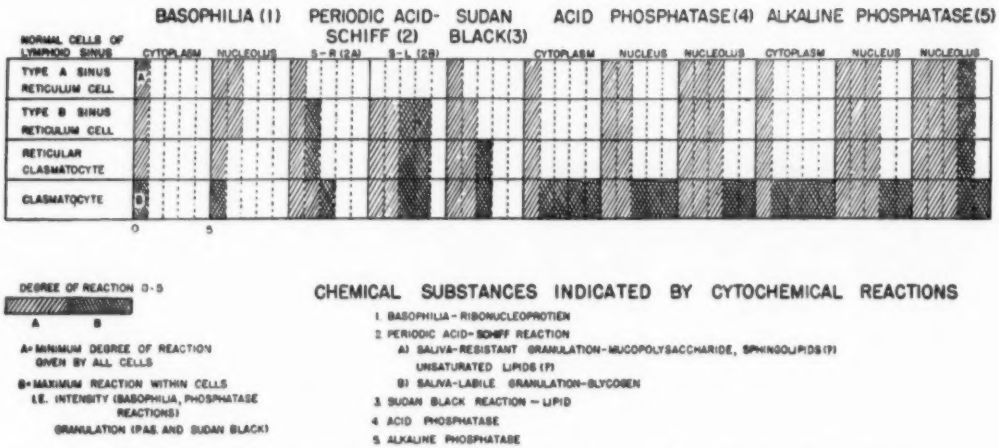
b) *Elongated reticulum cells of the sinuses:* (See text-figs. 1, 2, and 3.) Reticulum cells lining and extending across the lymphatic sinuses have been divided into two categories to facilitate their cytochemical description: 1) *Elongated reticulum cells* (*type "a"*) exhibiting cytochemical and morphologic characteristics identical with those of the reticulum cells comprising the lymph node stroma. 2) *Elongated reticulum cells* (*type "b"*), which occur in association with clasmatocytes and reticular clasmatocytes (to be described later) in the dilated lymphatic sinuses, and which are characterized by containing a large number of PAS-positive and glycogen granules. Sudanophilic granules are usually more numerous in the cytoplasm of the type "*b*" sinus reticulum cell than in the elongated reticulum cells of the stroma and sinuses (type "*a*") although the ribonucleoprotein and phosphatase content are similar in these cell forms. Morphologically, the type "*b*" sinus reticulum cell has a coarser cytoplasm with well-defined cell borders and a less vesicular nucleus than the type "*a*" sinus reticulum cell.

c) *Round reticulum cells:* (See text-figs. 1 and 3.) These cells have large, round nuclei with sparse chromatin, several medium-sized, irregularly shaped nucleoli, and a distinct nuclear membrane. The round reticulum cell has a moderate amount of "fluffy" cytoplasm and is colored lavender (+−++) following toluidine blue staining. Small PAS-positive and sudanophilic granules are localized about the cytocentrum; the latter may be scattered throughout the cytoplasm. Both acid and alkaline phosphatases are found in the cytoplasm, nucleus, and nucleoli of these cells. The alkaline phosphatase activity in the cytoplasm is +, the nuclei +++, and nucleoli ++++ (fig. 3). The acid phosphatase activity in the cytoplasm is +, the nucleus ++, and the nucleoli ++−+++ (fig. 4).

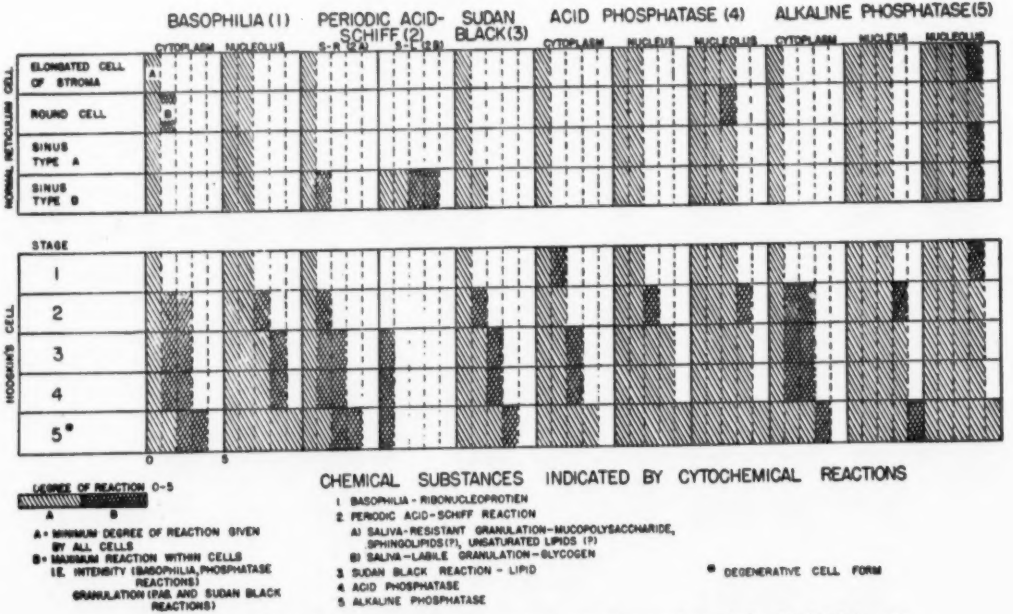
2) *Endothelial cells:* (See text-fig. 1.) The endothelial cells lining the capillaries and small venules of the lymph node vary greatly in size and shape and occasionally may be cuboidal rather than flattened. Their nuclei are more vesicular than the nuclei of the reticulum cells comprising the stroma. The cytochemical reactions employed yield similar reactions in both the small and the enlarged forms. The ribonucleoprotein content of the cytoplasm and the phosphatase activity of the cytoplasm, nucleus, and nucleoli are slightly greater in the endothelial cell than in the elongated reticulum cell. The cytochemistry of the endothelial cell of the blood vessels and the elongated reticulum cell are illustrated in text-figure 1.

3) *Lymphatic cells:* The lymphatic-cell series has been divided into four groups to facilitate cytochemical and morphologic description: *lymphoblasts*, considered to represent a lymphocyte progenitor; *large*, *intermediate*, and *small lymphocytes*.

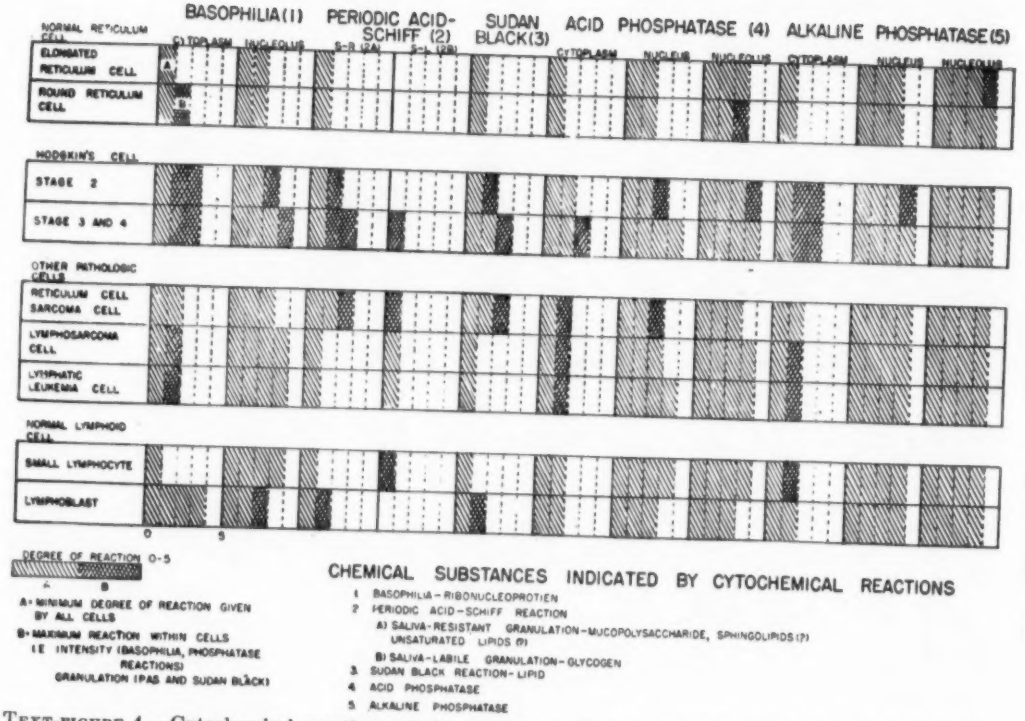
a) *Lymphoblasts:* (See text-fig. 1.) Lymphoblasts usually are large, round cells generally located in the secondary lymphatic nodules and



TEXT-FIGURE 2.—Cytochemical reactions of normal cells comprising the lymphoid sinuses.



TEXT-FIGURE 3.—Cytochemical reactions of normal reticulum cells and the Hodgkin's cell.



TEXT-FIGURE 4.—Cytochemical reactions of the normal reticulum cell and lymphoid cell and the cells of Hodgkin's disease, reticulum-cell sarcoma, lymphosarcoma, and lymphatic leukemia.

occasionally in other portions of the node. They may be differentiated from the round reticulum cell by their somewhat smaller size, denser chromatin, smaller number of nucleoli, and greater content (++++) of cytoplasmic ribonucleoprotein. The acid and alkaline phosphatase activity of the cytoplasm is slightly greater than that of the round reticulum cell. PAS-positive and sudanophilic granules may be more numerous in the lymphoblast than in the round reticulum cell. The cytochemical differences in these two cell forms are illustrated in text-figure 1.

b) *Small, intermediate and large lymphocytes:* (See text-figs. 1 and 4.) The small lymphocyte is a round cell with a dense irregular chromatin network and a small rim of cytoplasm surrounding the nucleus. The ribonucleoprotein of the cytoplasm of the small lymphocyte is colored blue or lavender (+) with toluidine blue. Lymphocytes with lavender or medium blue cytoplasm are considered to represent forms younger than those with light blue cytoplasm. A small number of PAS-positive and sudanophilic granules are concentrated in the nuclear hof. A few glycogen granules can be demonstrated in a small proportion of the lymphocytes. As will be pointed out later, the presence and amount of glycogen and mucopolysaccharide within the lymphocyte aid in the identification of abnormal forms. The cytoplasm of the small lymphocyte possesses a weak alkaline and acid phosphatase activity (+−++) and the nucleus a high alkaline (++++) and acid (+++−++++) phosphatase activity. Often one, but occasionally several, small dark areas (nucleoli) may be seen in the nucleus following the acid and alkaline phosphatase reactions.

In general, large and intermediate lymphocytes exhibit cytochemical reactions resembling those of the small lymphocyte, while the younger, more basophilic forms of these cells exhibit cytochemical reactions intermediate between the lymphoblast and the mature lymphocyte.

4) *Reticular clasmatocytes:* (See text-fig. 2.) All stages of transformation between reticular cells of the sinuses and reticular clasmatocytes have been observed morphologically and cytochemically. The reticular clasmatocytes are oval or triangular in shape, with irregular cytoplasmic outlines, and contain variable amounts of phagocytized material. The nuclei contain one or two small nucleoli and have a chromatin pattern closely resembling that of the type "*b*" sinus reticulum cell. A large amount of PAS-positive material as well as many sudanophilic granules fills the entire cytoplasm, forming a rosette about the cytocentrum. Glycogen and hemosiderin inclusions are often present. Hemosiderin particles are PAS-positive and vary in color from orange-red to deep red. Acid and alkaline phosphatase activity of the reticular clasmatocytes is similar to that of the reticulum cell of the sinus (type "*b*"), although frequently the cytocentrum exhibits a greater phosphatase activity. Phagocytized inclusions may exhibit alkaline phosphatase activity.

5) *Clasmatocytes:* (See text-fig. 2.) The clasmatocyte, which appears to arise from the reticular clasmatocyte, is small with indistinct cytoplasm

containing variable amounts of phagocytized material and a compact nucleus with irregular margins; nucleoli are usually absent. The cytochemistry of the clasmatocyte is similar to that of the reticular clasmatocyte, with the exception of occasional high phosphatase activity.

6) *Plasma cells, mast cells, and granulocytes:* The cytoplasm of the plasma cell becomes deeply basophilic and metachromatic following exposure to toluidine blue. The stainability of the cytoplasm is greatly reduced by prior digestion with ribonuclease. The PAS and Sudan black reactions color the entire cytoplasm a pale homogeneous pink and greenish-black, respectively (formol-Zenker fixation), and a few PAS-positive and sudanophilic granules are localized about the cytocentrum. The plasma cell cannot be distinguished from the lymphocyte by means of the alkaline and acid phosphatase reactions.

Mast-cell granules are sudanophilic, PAS-positive, metachromatic (toluidine blue), and contain a ribonucleoprotein component.

Neutrophils contain sudanophilic, glycogen, and PAS-positive granules and exhibit weak alkaline and acid phosphatase reactions. Eosinophil granules are sudanophilic and PAS-negative; the cytoplasm is PAS-positive.

Cells of the Lymphoblastomas

1) *Modified reticulum cells of Hodgkin's disease:* (See text-figs. 1 and 3.) Both cytochemical and morphologic examination of lymph nodes obtained from patients with Hodgkin's disease reveals transitional forms between normal elongated reticulum cells forming the stroma and sinuses of the lymph node and large definitive Hodgkin's cells [5] and their early and late degenerative forms. The cytomorphosis of the Hodgkin's cell is divided into six stages to facilitate cytochemical and morphologic description: 1) enlargement of the elongated reticulum cell forming the stroma and sinuses; 2) rounding of the reticulum cell; 3) continued enlargement resulting in a cell containing a large nucleus with sparse chromatin which frequently aggregates about the nuclear membrane and the nucleoli; 4) indentation and lobulation of the nucleus of the large mononuclear (Hodgkin's) cell and a concomitant enlargement of the cytocentrum; multinucleated cells may develop during this stage; 5) thickening of the chromatin, first at the nuclear membrane and nucleolar borders and later throughout the nucleus, wrinkling of the nucleus, and fragmentation of the nucleoli; and 6) karyorrhexis.

There is a gradual increase in nuclear and nucleolar acid phosphatase during the transformation from the normal reticulum cell to the stage 1 Hodgkin's cell. Although the increase may precede a morphologic modification, both changes usually occur concurrently.

Cytoplasmic ribonucleoprotein (fig. 5), PAS-positive and sudanophilic granules and acid and alkaline phosphatase activity are greater in the cells of stage 2 than stage 1.

The ribonucleoprotein component of the definitive Hodgkin's cell (stages 3 and 4) is greater than in the earlier forms (stages 1 and 2). The

[5] The term "Hodgkin's cell" is used throughout this study in place of the term "Reed-Sternberg cell."

ribonucleoprotein in the cytocentrum of the mature Hodgkin's cell is colored blue (+−++) and that of the remainder of the cytoplasm is colored lavender (+−+++) with toluidine blue (fig. 6). The basophilic component of the nucleoli is greatly reduced following ribonuclease digestion. Small PAS-positive and sudanophilic granules are distributed throughout the cytoplasm with a concentration of larger PAS-positive and sudanophilic granules about the cytocentrum (figs. 7 and 8). Glycogen has been found in widely varying amounts in Hodgkin's cells in 3 of 24 cases studied. In a few cells, a small number of large PAS-positive (fig. 7) and sudanophilic granules, having an irregular contour and exhibiting alkaline phosphatase activity (fig. 11), are localized at the periphery of the PAS-positive and sudanophilic rosette. The PAS and Sudan black reactions color the large nucleoli of the Hodgkin's cell. The acid and alkaline phosphatase activity of the definitive Hodgkin's cell is greater than that of the normal round reticulum cell and younger forms of Hodgkin's cells (stages 1 and 2), (text-fig. 3). Acid and alkaline phosphatase is demonstrated in the nucleus and nucleoli of the stage 3 and stage 4 Hodgkin's cell as ++++, while the alkaline phosphatase of the cytoplasm is variable (+−+++) (figs. 10 and 11); the acid phosphatase activity is ++ to +++ (fig. 12) with an occasional darker zone near the cytocentrum.

The nucleus of the degenerating Hodgkin's cell (stages 5 and 6) may stain so deeply during the final stages of cell degeneration that nuclear structure is obscured. The cytoplasmic ribonucleoprotein of the degenerating cell is altered and stains a muddy blue rather than lavender with toluidine blue. Neutral fat vacuoles may develop in the cytoplasm of the degenerating Hodgkin's cell (*21*); the cytoplasm near the vacuole is PAS-positive. PAS-positive and sudanophilic granules and acid (fig. 14) and alkaline phosphatase activity are increased in the degenerating cells. Hodgkin's cells may undergo degeneration at any point during their development after having become round (stage 2).

In a few instances, the first sign of degeneration is indicated by a diminution or absence of the nucleolar ribonucleoprotein and phosphatase activity (fig. 15). Only the nucleolar rim remains distinguishable, and the phosphatase activity of the cytoplasm and nucleus is not as great as that observed in the other degenerating forms.

Neutrophils and eosinophils may aggregate around the large nuclei of degenerating Hodgkin's cells in which the cytoplasm and nucleoli are absent. The nuclei appear to be undergoing karyolysis and are poorly stained following the nucleal and toluidine blue reactions.

The amount of desoxyribonucleoprotein in the nuclei of many definitive Hodgkin's cells appears to be greater than that of the normal reticulum cell and the stage 1 Hodgkin's cell. This concept is based upon a visual comparison of the size of the nucleus, chromatin distribution, and the thickness of the chromatin strands of these cell forms (figs. 6 and 16).

Multinucleated Hodgkin's cells exhibit cytochemical reactions that

appear to depend upon their developmental stage and resemble those reactions indicated for the mononuclear Hodgkin's cell.

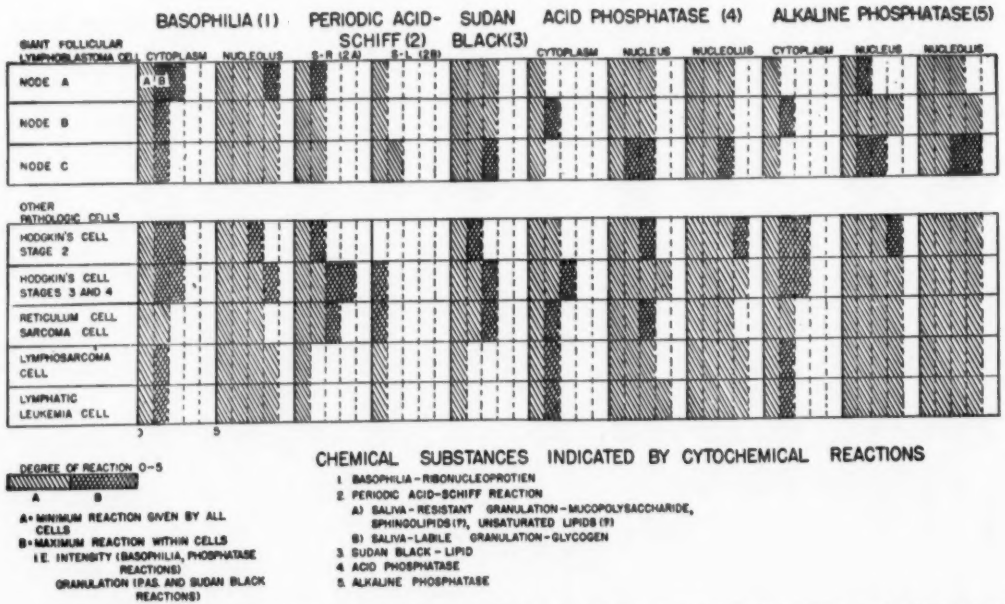
2) *Modified reticulum cells of reticulum-cell sarcoma:* (See text-figs. 1 and 4.) Modified cells of reticulum-cell sarcoma resemble normal, round reticulum cells and stage 2 Hodgkin's cells in size and contour. The vesicular nucleus contains two to five medium-sized nucleoli. The cytoplasmic ribonucleoprotein is colored lavender (++) and the nucleoli appear blue (+++) following exposure to toluidine blue. The cytoplasm of this cell is colored a pale homogeneous pink and greenish-black following the PAS and Sudan black reactions (formol-Zenker fixation). PAS-positive and sudanophilic granules are distributed throughout the cytoplasm and localize about the cytocentrum. Glycogen is demonstrable in many reticulum-cell sarcoma cells. The alkaline phosphatase activity resembles that of the stage 2 Hodgkin's cell and is greater in the reticulum-cell sarcoma cell than in the normal round reticulum cell, lymphoblast, and lymphosarcoma cell. The acid phosphatase activity of the reticulum-cell sarcoma cell resembles the phosphatase activity of the cells mentioned above. Using the techniques described in this report, the reticulum-cell sarcoma cell cannot be distinguished cytochemically from the stage 2 Hodgkin's cell, but may be differentiated from definitive Hodgkin's cells (stages 3 and 4), (text-fig. 4).

3) *Modified lymphocytes observed in chronic lymphatic leukemia and lymphosarcoma:* (See text-figs. 1 and 4.) Morphologically, the modified lymphocytes of chronic lymphatic leukemia resemble normal lymphocytes, although the normal lymphocyte presents a more dense chromatin pattern. The lymphosarcoma cell has a more vesicular nucleus than either the normal or the leukemic lymphocyte. In spite of these differences, it is often difficult to distinguish lymphatic hyperplasia, lymphatic leukemia, and lymphosarcoma on a morphologic basis. Modified lymphocytes of chronic lymphatic leukemia and lymphosarcoma may possess a more basophilic cytoplasm than do normal mature lymphocytes.

Many leukemic and sarcomatous lymphocytes contain more PAS-positive and glycogen granules than do normal lymphocytes, although the number of granules was not increased in one of nine cases of lymphatic leukemia studied.

The sudanophilic granulation of the leukemic and sarcomatous lymphocytes does not appear to differ from that of the normal lymphocyte. The alkaline and acid phosphatase activity of the nucleus of the leukemic lymphocyte resembles that of the normal lymphocyte, while the phosphatase activity of the nucleus of the sarcomatous lymphocyte is less than that of the normal lymphocyte. Following the alkaline and acid phosphatase reactions, one large nucleolus is usually demonstrable in the leukemic lymphocyte and several small nucleoli in the sarcomatous lymphocyte. The cytochemical reactions of the sarcomatous and leukemic lymphocytes may be compared with those of normal lymphocytes in text-figure 4.

4) *Cells of giant follicular lymphoblastoma:* (See text-figs. 1 and 5.)



TEXT-FIGURE 5.—Cytochemical reactions of the cells of giant follicular lymphoblastoma and the cells of other lymphoblastomas.

Lymph node cells obtained from three patients with giant follicular lymphoblastoma have given inconsistent cytochemical reactions and present a variable cellular morphology.

Examination of Node A reveals that the cells exhibit morphologic characteristics resembling those of the lymphosarcoma cell; cells of Node B resemble the reticulum-cell sarcoma cell; and cells of Node C exhibit morphologic variations between the reticulum-cell sarcoma cell and the lymphosarcoma cell. Sudanophilic and PAS-positive granules are distributed throughout the cytoplasm; glycogen is present in variable amounts in the cells of each node. The acid and alkaline phosphatase activity of the cells of Nodes A and C is less than that of the cells of Node B; however, the alkaline phosphatase activity of the cells of Node B closely resembles that of the reticulum-cell sarcoma cell. The cytochemistry of the cells of giant follicular lymphoblastoma may be compared with that of other lymphoblastoma cells in text-figure 5.

5) *Monoblasts and young monocytes of acute monocytic leukemia:* (See text-fig. 1.) Monoblasts and young monocytes are characterized by round or slightly indented vesicular nuclei with one or two nucleoli. The ribonucleoprotein of the cytoplasm of these leukemic cells is colored lavender (+−++) with toluidine blue. A small number of PAS-positive and sudanophilic granules form a rosette about the cytocentrum. Following the PAS reaction the cytoplasm of the leukemic monocyte is colored a faint homogeneous pink; this reaction of the cytoplasm is not changed following previous treatment with ribonuclease or hyaluronidase. The alkaline and acid phosphatase activity of the leukemic monocyte is less than that of the lymphosarcoma cell (see text-fig. 1).

Modified Cells of Miscellaneous Human Lymph Nodes

1) *Modified reticulum cells of Boeck's sarcoid:* (See text-fig. 1.) Lymph nodes obtained from patients with Boeck's sarcoid contain "tubercle-like" masses of modified cells surrounded by dense concentric rings of reticular fibers. Elongated reticulum cells, lymphocytes, plasma cells and mast cells are enmeshed between these fibers and throughout the node.

Reticulum cells of the stroma outside the rings of reticular fibers exhibit normal morphologic and cytochemical characteristics. The modified reticulum cells within the rings of fibers exhibit an increase in cytoplasmic ribonucleoprotein (+−++) and in PAS-positive and sudanophilic granules; alkaline phosphatase activity is increased in both the cytoplasm (+++) and nucleus (++++). Following migration into the "tubercle-like" granuloma, the modified reticulum cell becomes smaller and more irregular in shape. The amount of cytoplasmic ribonucleoprotein and alkaline phosphatase decreases, although the amount of PAS-positive and sudanophilic material is increased. This cell then exhibits a high acid phosphatase activity (+++++), especially in the region of the cytocentrum. Text-figure 1 indicates the cytochemical reactions exhibited by the mononuclear cell of Boeck's sarcoid located in the "tubercle-like" formation.

Giant multinuclear cells of Boeck's sarcoid vary greatly in size and in the number of nuclei. There is a concentration of PAS-positive and sudanophilic granules in the region of the cytocentrum as well as a less dense distribution throughout the cytoplasm. Following exposure to toluidine blue, the ribonucleoprotein in the region of the cytocentrum is colored blue (+), while the remainder of the cytoplasm is colored blue-lavender (++-+++). The alkaline phosphatase activity of the giant cell is not marked; however, acid phosphatase activity is very high, especially in the region of the cytocentrum (+++++-++++++). Giant cells in the initial stages of degeneration exhibit higher alkaline phosphatase activity and have denser chromatin than the mature forms of giant cells.

Shaumann bodies, found in the cytoplasm of the giant cells, are metachromatic (deep purple) with toluidine blue, PAS-positive, and possess a high acid and alkaline phosphatase activity.

2) *Modified lymphocytes (Rieder cells) of infectious mononucleosis:* (See text-fig. 1.) Large and intermediate size Rieder cells exhibit a moderately basophilic vacuolated cytoplasm and deeply indented nuclei. A small number of PAS-positive and sudanophilic granules are distributed throughout the cytoplasm and are localized in the region of the cytocentrum; glycogen may also be present in these cells. Acid and alkaline phosphatase preparations were inadequate for cytologic study.

3) *Metastatic carcinoma cells involving the lymph node:* The cytochemical reactions exhibited by carcinoma cells obtained from a small number of individuals have been variable, although glycogen is present in the carcinoma cells of all nodes examined. The cytochemistry of these cells may depend upon their primary site of origin (*10, 18, 22*); therefore, the cytochemical evaluation has been postponed awaiting further material.

DISCUSSION

Cytochemical changes occur in cells of normal human lymph nodes during maturation and development. These changes are evident in the transformation of elongated reticulum cells into clasmatocytes by the elaboration of large amounts of PAS-positive material (text-fig. 2); during the formation of the lymphoblast from the round reticulum cell by the increase in ribonucleoprotein and phosphatase activity (text-fig. 1); and during lymphocyte maturation by a decrease in ribonucleoprotein, mucopolysaccharide, and lipid granules and a slight increase in nuclear acid phosphatase.

It is possible to describe five general cytochemical modifications taking place during differentiation and maturation of neoplastic and nonneoplastic lymph node cells. In order to make this classification, it is necessary to assume that the elongated reticulum cell [6] is the basic prototype from which other lymph node cells are derived. The cytochemical modifications are 1) A gradual increase in cytoplasmic ribonucleoprotein

[6] The elongated reticulum cell referred to includes both those of the stroma and sinuses (type "a") which exhibit similar cytochemistry and morphology.

and a similar increase in phosphatase activity, first in the nucleolus, then in the cytoplasm and nucleus. This type of cytochemical modification takes place during the formation of (a) the lymphoblast from the elongated reticulum cell through the intermediate round reticulum cell stage; (b) the plasma cell; (c) the early stages of Hodgkin's cells (stages 1 and 2); and (d) possibly the early stages of the reticulum-cell sarcoma cell. The formation of ribonucleoprotein and the increase in phosphatase activity suggest an increase in cell metabolism and a higher rate of protein synthesis. 2) An elaboration of PAS-positive and sudanophilic granules with a continued increase in ribonucleoprotein content and phosphatase activity. Cells in this group are the later stages in the Hodgkin's cell (stages 3–5) and the reticulum-cell sarcoma cell. 3) The elaboration of PAS-positive and sudanophilic granules without an increase in ribonucleoprotein occurring with or without an increase in phosphatase activity. Cell forms included in this group are (a) the transformation stages between the sinus reticulum cell and clasmatocyte and (b) the formation of the modified reticulum cell of Boeck's sarcoid. 4) A decrease in ribonucleoprotein and an increase in phosphatase activity. This modification is observed during the maturation of the lymphocyte from the lymphoblast. 5) An increase in phosphatase activity and an elaboration of glycogen. Cells in this group include the lymphocytes of lymphatic leukemia and lymphosarcoma.

Definitive Hodgkin's cells (stages 3 and 4) contain more lipid and mucopolysaccharide granules, more ribonucleoprotein, and have a higher acid and alkaline phosphatase activity than normal elongated and round reticulum cells (text-fig. 3). The first discernible cytochemical change occurring during the formation of the Hodgkin's cell is a gradual increase in acid phosphatase of the nucleus and nucleolus, followed by an increase in the ribonucleoprotein of both the cytoplasm and nucleolus and an elaboration of lipid and mucopolysaccharide granules. Acid and alkaline phosphatase activity continue to increase as the cell becomes more mature and undergoes degeneration. The amount of desoxyribonucleoprotein in the nucleus of many large Hodgkin's cells appears greater than that of normal reticulum cells and early forms of Hodgkin's cells.

Progressive enlargement of the nucleolus and the "nucleolus-associated chromatin" of the Hodgkin's cell is followed by a progressive increase in ribonucleoprotein in the cytoplasm and nucleolus. This type of change has been described as a characteristic of rapidly growing cells (*23*). According to Caspersson (*23*), the "nucleolus-associated chromatin" elaborates ribose nucleotide and protein substances rich in diamino acids during protein synthesis. It is postulated that nucleoli are formed and enlarged by the accumulation of these substances. In this manner the "nucleolus-associated chromatin" may regulate nucleic acid metabolism and indirectly regulate protein metabolism. Caspersson further postulates that the endocellular mechanism, normally inhibiting the activity of the protein-forming system, is almost nonfunctional in carcinoma and sarcoma cells, resulting in a specific cytochemical change as compared with non-neoplastic cells. Ultraviolet analysis of giant follicular lymphoblastoma

cells (exhibiting changes suggestive of reticulum-cell sarcoma) are said to indicate a "continuous change in the activity of the system for protein formation" (*23*).

Phosphatase activity in the Hodgkin's cell appears to vary directly with certain phases of cellular metabolism. Since the early elaboration of phosphatase in the nucleolus and nucleus occurs during differentiation and just prior to the formation of cytoplasmic ribonucleoprotein, it is possible that these enzymes may function in the phosphorylation of ribonucleoprotein. Cytochemical observations offer evidence that degeneration of the Hodgkin's cell may be manifested in two ways: 1) loss of phosphatase activity, resulting in an inability to synthesize nucleoprotein and specific cell proteins and 2) degradation and alteration of nucleoprotein as well as other chemical constituents of the cell by the phosphatase system. Ross (*24*) demonstrated that alkaline phosphatase acts very slowly on highly polymerized desoxyribose nucleic acid. The effect of the enzyme becomes greater as the molecular weight of the nucleic acid decreases, presumably due to the action of the enzyme on the more numerous terminal phosphate groups available as a result of depolymerization. Further examination of phosphatase specificity employing a variety of substrates is necessary in order to establish their role in cellular metabolism.

The division of Hodgkin's disease into three histopathologic types (*25*) does not seem warranted from a cytochemical point of view. This view is in agreement with that of Slaughter and Craver (*26*), who have objected to Jackson and Parker's (*25*) classification because lymph nodes observed in one individual may exhibit all stages of cytophysiologic variation described by the latter; Reed (*27*) and Longcope (*28*) have observed similar variations in a group of nodes removed from a single region. Contributions to the understanding of the Hodgkin's cell prior to 1947 are discussed in a review by Hoster and Dratman (*29*). Cytochemical evidence described in this report supports the view of other investigators that the reticulum cell gives rise to the Hodgkin's cell (*21, 30*) and does not substantiate the theory that they arise from reticular macrophages (*31*).

Using the cytochemical techniques described, modified reticulum cells of Hodgkin's disease (stage 2) cannot be distinguished from the reticulum-cell sarcoma cell (text-fig. 4). The Hodgkin's cell (stages 2–4) and the reticulum-cell sarcoma cell contain more mucopolysaccharide and lipid granules than do leukemic and sarcomatous lymphocytes (text-fig. 4). Although glycogen is rarely present in the Hodgkin's cell, it is frequently found in the reticulum-cell sarcoma cell and is usually present in modified lymphocytes of lymphosarcoma and chronic lymphatic leukemia. The phosphatase activity of these cell forms differs only slightly (text-fig. 4).

Many of the modified lymphocytes of lymphosarcoma, chronic lymphatic leukemia, and infectious mononucleosis contain more glycogen and mucopolysaccharide granules than do normal lymphocytes (text-fig. 4). Wislocki *et al.* (*32*) and Gibb and Stowell (*33*) report more glycogen in leukemic lymphocytes observed in blood films than in normal lymphocytes. Modified lymphocytes of chronic lymphatic leukemia often

exhibit a higher nuclear phosphatase activity than do lymphosarcoma cells. The presence of a large, single nucleolus in leukemic lymphocytes distinguishes these cells from sarcomatous lymphocytes, which contain several small nucleoli. All normal lymphocytes show alkaline phosphatase (and acid phosphatase) activity as well as those of chronic lymphatic leukemia and lymphosarcoma. This observation differs from those of Wachstein (*11*) and Kabat and Furth (*10*).

Cells occurring in lymph nodes with lymphoblastoma involvement but morphologically presumed to be nonneoplastic exhibit normal cytochemical reactions. This observation provides evidence that cellular hyperplasia resulting in certain disease conditions (*i. e.*, eosinophilia frequently noted in Hodgkin's disease, proliferation of plasma cells and clasmatocytes in infectious diseases and nonspecific reticulum-cell and lymphatic hyperplasia) is a secondary or modulating manifestation rather than a neoplasia of these cell forms.

The presence of two forms of ribonucleoprotein is offered in explanation of the blue and lavender reactions elicited by the cytoplasm of lymph node cells following exposure to toluidine blue, although a positive metachromatic reaction may be produced if the ratio of dye to nucleic acid is high (*34*). In cells undergoing protein synthesis the lavender color prevails, suggesting the presence of a less highly polymerized form of nucleic acid than that giving rise to the blue color. The occurrence of more than one form of ribonucleoprotein (*35*) and ribonuclease (*36*) has been reported.

The chemical nature of the cytoplasmic and nucleolar material that exhibits a deeply basophilic color and a homogeneous coloration following the PAS and Sudan black techniques suggests the presence of a ribonucleoprotein-lipid complex in which the lipid fraction contains an unsaturated fatty acid; the existence of a carbohydrate in the moiety cannot be ruled out. The close agreement in the distribution and amount of sudanophilic and PAS-positive granules suggests that a lipid-carbohydrate (mucopolysaccharide) complex may exist in these granules, or that the PAS reaction is demonstrating an unsaturated lipid or sphingolipid.

The data presented have provided evidence that normal and abnormal lymph-node cells have cytochemical individuality and that cytochemical alterations may take place in advance of discernible morphologic changes; in most instances these changes appear to be quantitative rather than qualitative as ascertained by the relative intensity of the reactions and the amount of cytoplasmic granulation. Such differences have been remarkably consistent among similar cell forms and during the maturation of a cell lineage and undoubtedly reflect differences in metabolic activity in the various cell forms. These preliminary studies suggest that cytochemical techniques may prove to be valuable in the differentiation of the lymphoblastomas.

SUMMARY

1. An attempt has been made to characterize the cells of normal and pathologic human lymph nodes. Stages in the formation and development of these cells have been demonstrated cytochemically and morphologically.

2. Evidence suggests that in cells of lymph nodes cytochemical changes occur prior to discernible morphologic differentiation. These cytochemical changes appear to be quantitative rather than qualitative in nature.

3. Cytochemical evidence presented supports the view that the reticulum cell gives rise to the Hodgkin's cell.

4. Cytochemical differences between cells of normal lymph nodes and the modified cells of Hodgkin's disease, reticulum-cell sarcoma, lymphosarcoma, lymphatic leukemia, and Boeck's sarcoid are described.

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PLATES

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PLATE 41

FIGURE 1.—Normal elongated reticulum cells (A) and small lymphocytes (B). Alkaline phosphatase. × 1,250.

FIGURE 2.—Normal elongated reticulum cells (A) and small lymphocytes (B). Acid phosphatase. × 1,250.

FIGURE 3.—Normal round reticulum cell (A), elongated reticulum cell (B) and small lymphocytes (C). Alkaline phosphatase. × 1,250.

FIGURE 4.—Normal round reticulum cell (A) and small lymphocytes (B). Acid phosphatase. × 1,250.

FIGURE 5.—Developing Hodgkin's cells, stages 1 (A), 2 (B), and 3 (C) and small lymphocytes. Toluidine blue. × 1,250.

FIGURE 6.—Definitive stage 3 Hodgkin's cell with prominent region of the cytocentrum (A). Toluidine blue. × 1,250.

FIGURE 7.—Definitive stage 3 Hodgkin's cell (A) with fine PAS-positive granules scattered throughout the cytoplasm and concentrated in the region of the cytocentrum. One Hodgkin's cell (B) contains large PAS-positive granules. PAS reaction, reticular fibers (C) exhibit a strong reaction. × 1,250.

FIGURE 8.—Stage 3 Hodgkin's cells with sudanophilic granules scattered throughout the cytoplasm and localized about the nucleus and cytocentrum. Sudan black. × 1,250.

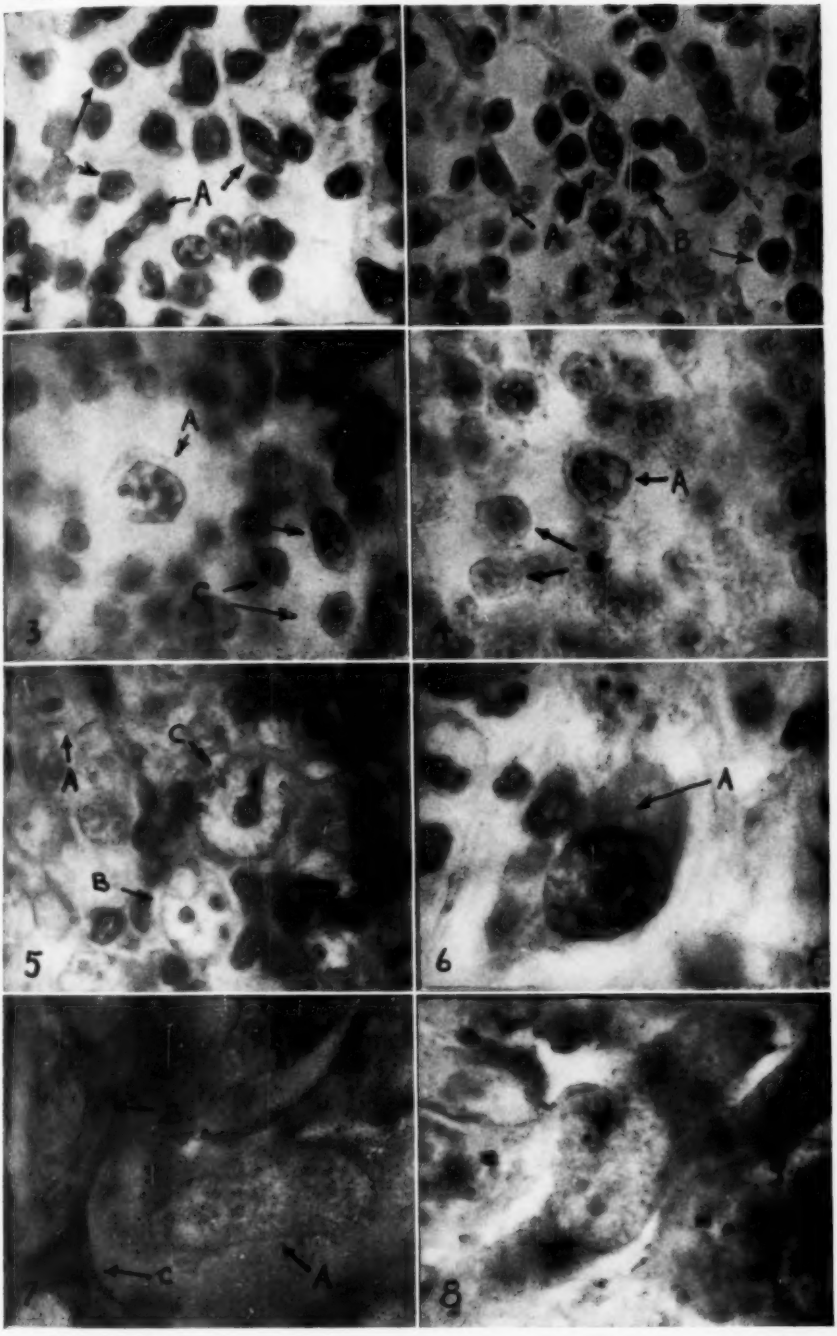

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PLATE 42

FIGURE 9.—Stage 1 (A) and 2 (B) Hodgkin's cells following exposure to the alkaline phosphatase reaction. × 1,250.

FIGURE 10.—Binucleated Hodgkin's cell following the alkaline phosphatase reaction. × 1,250.

FIGURE 11.—Multinucleated Hodgkin's cell demonstrated by the alkaline phosphatase reaction. Note the lighter coloration of the cytoplasm about the cytocentrum and the small darker bodies located in this region. × 1,250.

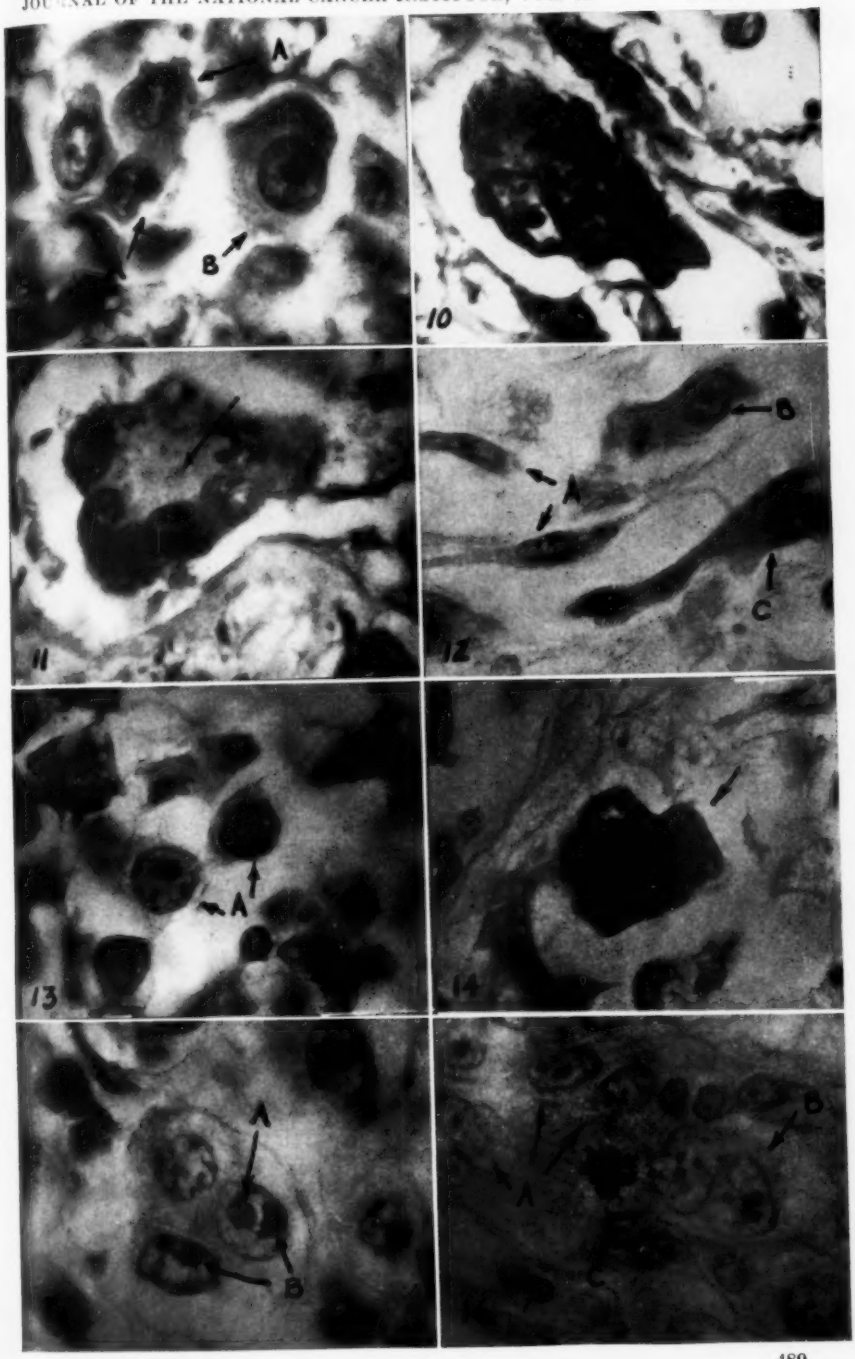
FIGURE 12.—Stage 1 (A), 2 (B), and 3 (C) Hodgkin's cells. Acid phosphatase. × 1,250.

FIGURE 13.—Stage 2 Hodgkin's cells (A). Acid phosphatase. × 1,250.

FIGURE 14.—High acid phosphatase reaction in the degenerating Hodgkin's cell. Acid phosphatase. × 1,250.

FIGURE 15.—Trinucleate Hodgkin's cell illustrating the decrease in acid phosphatase activity in some degenerating cells. Note that there is a single lightly colored nucleolus (A) and several darkly colored nucleoli (B) in this cell. Acid phosphatase. × 1,250.

FIGURE 16.—Differentiating Hodgkin's cells, stage 1 (A); binucleate (B), and mitotic forms (C). Note the apparently greater amount of desoxyribonucleoprotein in the nucleus of the large Hodgkin's cell as compared with the younger forms. Nucleal reaction. × 1,250.


A
B
10
11
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C
12
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C

HISTOPATHOGENESIS OF SQUAMOUS-CELL CARCINOMA INDUCED IN THE FORESTOMACH OF MICE BY INTRAMURAL INJECTION OF 20-METHYLCHOLANTHRENE [1]

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Squamous-cell carcinoma of the forestomach has been induced in mice by the oral administration and by the intramural injection of certain of the polycyclic carcinogenic hydrocarbons (*1*, *6*). The morbid anatomy, histopathology, and histopathogenesis of this lesion have been described in mice ingesting 20-methylcholanthrene and 1,2,5,6-dibenzanthracene in aqueous oil emulsions (*6*). The present report deals with a similar study of squamous-cell carcinoma of the forestomach induced in mice by a single intramural injection of 20-methylcholanthrene with emphasis on the features of histopathogenesis (*7*).

MATERIALS AND METHODS

Mice of two genetic types were employed, consisting of 110 strain C57 brown and 110 A backcross hybrid animals, each type equally divided as to sex. They were from stocks maintained in this Institute in which previous experiments had shown animals of these genetic types to be susceptible to the induction of squamous-cell carcinoma of the forestomach. The A backcross hybrid mice were obtained by mating strain A females to strain C57 black males and backcrossing the females of the F_1 generation to male strain A mice. The animals were 3 to 10 months of age when selected for the experiment and they were maintained on a diet of Purina laboratory chow and tap water.

Using intraperitoneal nembutal anesthesia supplemented by ether inhalation as required, the stomach was delivered through a left paramedian incision. A short-bevel, 27-gauge needle attached to a tuberculin syringe was inserted tangential to the serosal surface of the anterior wall of the forestomach at a point 2 to 3 mm. proximal to the limiting ridge. The needle was continued obliquely and superiorly to the left for a distance of 2 to 4 mm., at which point approximately 0.02 ml. of an aqueous suspension containing 0.6 mg. of 20-methylcholanthrene (table 1) was deposited in the submucosa. This produced a small bleb 3 mm. in diameter, which remained after withdrawing the needle. Following injection, the stomach was returned to the peritoneal cavity and the abdominal wound was closed.

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TABLE 1.—*Composition of aqueous 20-Methylcholanthrene suspension* [1]

	Grams, percent
20-methylcholanthrene	3.0
Aerosol O T	.01
Methocel (4,000)	.2
Ethyl alcohol (distilled water—q. s. 100)	1.0

pH adjusted to 7.4 with 0.1 N NaOH prior to making up to volume with water.

[1] Prepared by Dr. Egon Lorenz, Chief, Biophysics Section, this Institute.

One mouse of each genetic type was killed by cervical dislocation approximately every 3½ days for 2½ months, and thereafter every 7 days for 12 months. Occasionally mice that died or that appeared moribund were substituted for those scheduled to be killed. The remaining mice were killed at the 77th week, when the experiment was terminated, unless during the interval they had developed large gastric tumors or appeared moribund, whereupon they were killed in order to obtain tissue free from post-mortem autolysis. The effective number of experimental mice was reduced from 220 to 165 by subtracting the animals that died post-operatively and those the tissues of which showed advanced post-mortem autolysis.

Twenty control mice (10 C57 brown and 10 A backcross animals) were given an injection of a control suspension identical with the experimental suspension except that it contained no 20-methylcholanthrene. Four control mice died within the first 3 months, providing control material for that period, and 11 died or were killed during the next 3½ to 17 months. Five were lost to the experiment because of post-mortem autolysis.

At autopsy the stomach was rapidly removed from the body, opened along the lesser curvature, spread out and pinned to a flat piece of cork, and fixed. A careful examination was made for metastases, implants, and other secondary neoplastic deposits. The tissues were fixed in Zenker-acetic-acid solution and occasionally in 10 percent formalin. They were embedded in paraffin, cut, and stained with hematoxylin and eosin. The sections of forestomach were routinely stained with, or by, hematoxylin and eosin (H and E), van Gieson's (VG), periodic-acid-Schiff's (PAS) (with and without previous diastase), phosphotungstic acid hematoxylin (PTAH), toluidine blue (TB) and crystal violet (CV), and were impregnated with silver by Wilder's or Laidlaw's methods for reticulin. Multiple sections were usually cut from the blocks of forestomach and serial sections were cut from selected blocks. Frozen sections were cut from a few formalin-fixed specimens of forestomach and examined under the polarizing microscope.

RESULTS

The mice, 165 in number, are classified into 2 major groups (table 2) according to the nature of the lesion of the forestomach. The precancerous lesions, 66 in number (group 1) are further subdivided into those with (22 cases) and those without (44 cases) a diverticulum. There

TABLE 2.—*Precancerous lesions and tumors of forestomach in mice following intramural injection of 20-methylcholanthrene*

	Days after injection														
	0-28	29-56	57-84	85-112	113-140	141-168	169-196	197-224	225-252	253-280	281-308	309-336	337-364	365-539	Total
Group 1															
Mice with precancerous lesions:															
Without diverticulum	14	9	8	3	2	2	1	1	0	0	0	0	0	4	44
With diverticulum	2	7	3	4	1	2	0	0	0	0	0	0	0	3	22
Group 2															
Mice with malignant tumors:															
Squamous-cell carcinoma	0	0	2	5	15	18	15	6	1	8	4	2	1	6	83
Sarcoma	0	0	0	0	0	[1] 2	0	[1] 2	0	0	0	0	0	0	4
Both carcinoma and sarcoma	0	0	0	1	4	4	1	0	0	0	0	0	0	2	12
Mice autopsied	16	16	13	13	22	28	17	9	1	8	4	2	1	15	165

[1] These specimens also showed precancerous changes.

are 4 additional specimens showing precancerous changes listed (table 2, footnote 1) under sarcoma of Group 2.

The malignant tumors, 99 in number (group 2), consist of 83 squamous-cell carcinomas, 4 sarcomas, and 12 mixed tumors (both squamous-cell carcinoma and sarcoma). A malignant gastric tumor is defined as a neoplastic lesion that infiltrates all coats of the viscus and forms a tumor mass on the serosa (*6*). Neoplastic lesions that did not achieve the magnitude consistent with carcinoma as defined are classified in the precancerous group. The first squamous-cell carcinoma of the forestomach was found in a mouse killed 60 days following injection. Of 132 animals autopsied 60 days or more following the injection, 95 or 73 percent showed squamous-cell carcinoma of the forestomach either alone or in association with sarcoma.

Statistical analysis [4] of the data collected under the experimental conditions as outlined, for evaluation of the incidence of squamous-cell carcinoma, sarcoma, and mixed carcinoma and sarcoma by sex, by genetic type, and by sex within each genetic type revealed no significant differences, with the exception of a slightly lower incidence of squamous-cell carcinoma in strain C57 brown female mice.

SARCOMA

The forestomachs with sarcoma often showed ulceration and much of the wall of the viscus replaced by the neoplasm, which was usually large and bulky and which occasionally extended to adjacent structures and organs, including liver, spleen, pancreas, peripancreatic lymph nodes, and lateral abdominal wall. In one case there were nodular implants of sarcoma on the pelvic peritoneum. Microscopically the sarcomas were generally composed of spindle-shaped cells arranged in interlacing bundles (figs. 7 and 8) containing collagen and reticulum in intimate relationship with the tumor cells. Fibroglia was demonstrated in only a few tumors and myoglia not at all, although a diagnosis of leiomyosarcoma was occasionally entertained. Huge, bizarre, single and multinucleated tumor giant cells were sometimes observed (figs. 7 and 9).

In the muscularis and submucosa of a few specimens of forestomach there were focal areas of atypical cells, which did not appear to be epithelial cells and which were suggestive of early sarcomatous transformation (fig. 10). These areas were composed of interlacing, irregularly oriented, small bundles and groups of enlarged spindle-shaped cells showing more than the usual variation in nuclear size, shape, and staining. Two of these foci occurred in forestomachs showing squamous carcinoma, and conceivably could have represented areas of an unusually active stroma supporting the epithelial tumor. Another focus bordered upon a peritoneal abscess, the result of perforating carcinoma.

In 12 of the 16 animals with sarcoma there was also an associated squamous-cell carcinoma either partially or entirely separated from the sarcoma or intimately blended with it as carcinosarcoma (fig. 7).

SQUAMOUS-CELL CARCINOMA

The carcinomas presented the same gross characteristics as those previously described (*6*) and arose in relation to the site of injection of the methylcholanthrene (fig. 1). Their histologic characteristics varied moderately with respect to cell variation and amount of keratin in different tumors and different portions of the same tumor, but all were classifiable

[4] Statistical analysis by M. A. Schneiderman, Biometrics Section of this Institute.

as squamous-cell carcinoma. A gland-like pattern occurred in one tumor but, since the acinous-like spaces appeared to be lined by squamous cells, and there was no associated mucus (PAS), the lesion was classified as squamous-cell carcinoma.

All the carcinomas completely invaded the wall of the stomach, extended laterally within the wall in all directions, and at times secondarily infiltrated the mucous membrane of both chambers. After penetrating the serosa, the tumors in 53 cases invaded adjacent organs and structures, including spleen, liver, pancreas, omentum, lymph nodes, diaphragm, and abdominal wall. Metastatic deposits were observed on the peritoneum in 3 animals (fig. 2), in the abdominal and mediastinal lymph nodes in 2 (fig. 3), in the lungs in 2 (fig. 6), and in the liver in 4 (fig. 5). In 13 additional mice, the liver contained multiple foci of atypical cells which, although resembling neoplastic epithelial cells (fig. 4), showed no associated keratin. Foci of atypical cells, indistinguishable from these, were observed associated with unquestionable metastases. Such foci did not occur in the liver of control mice or of the experimental mice without carcinoma.

TRANSPLANTATION

Out of 8 attempts at transplantation, 6 squamous-cell carcinomas and 2 sarcomas were successfully transplanted subcutaneously to other mice. No attempt was made to transplant lesions developing earlier than 13 weeks following injection.

PRECANCEROUS AND ASSOCIATED LESIONS

Changes in stroma, blood vessels, and muscle: The fluid component of the aqueous suspension was rapidly absorbed from the injection site, leaving behind a deposit of crystalline methylcholanthrene in the submucosa. An acute inflammatory reaction developed in and about this focus during the first 3½ days (fig. 12). This was characterized by the presence of mononuclear and polymorphonuclear leukocytes and a few fibroblasts. The cells, the walls of the blood vessels, and the connective-tissue fibers in the center of the focus became necrotic early. There were also degeneration and necrosis of the neighboring muscularis mucosae and of the collagenic fibers of the lamina propria above the injection site, while below it the muscularis propria appeared thickened and the adjacent muscle fibers degenerated. Immediately around the necrotic focus, the collagenic fibers of the submucosa were swollen, fragmented, angular, granular, and strongly PAS-positive. These alterations gradually became less pronounced as the distance from the inflammatory zone increased. However, for some little distance beyond the immediate area of the inflammatory focus, the collagenic fibers stained poorly with PAS and PTAH. Here also, the submucosa presented a pale eosinophilic, homogeneous appearance, suggesting the presence of proteinaceous material filling the intercollagenous spaces (figs. 15 and 26). The walls of the blood vessels stained deep pink with PAS.

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After 7 to 10 days, much of the necrotic material in the center of the focus had undergone liquefaction (fig. 13), and there was phagocytosis of methylcholanthrene crystals by macrophages, which had increased in number. Surrounding this central focus were macrophages and scattered fibroblasts embedded in a hyaline matrix, which stained deep red with eosin and dull pink with PAS. The small blood vessels in this region were often thickened and sometimes showed acute thromboangiitis. The lamina propria was edematous, particularly immediately below the hyperplastic mucosa, and there was beginning lymphangiectasia and telangiectasia, which increased with time. The area of damage in the muscularis propria increased in extent during the first week and showed patchy hyaline changes and fuchsinophilia (PAS). The adjacent muscle bundles became thickened and somewhat disarranged. Local peritoneal fibrosis ensued upon the extension of the inflammatory process to involve the serosa.

At 3 weeks, polymorphonuclear leukocytes were often prominent numerically in the exudate around the central focus containing the methylcholanthrene (fig. 14). The walls of many blood vessels at this time and subsequently showed irregular thickening, degeneration, and hyalinization (? fibrinoid degeneration). The involved portions of the vessel wall stained bright pink with PAS with and without prior treatment with diastase. Many vessels showed thromboangiitis. The injected crystalline material (fig. 14) became less evident after the first month (fig. 15), although in at least one specimen crystals were present 70 days postinjection. In animals autopsied later, multinucleated foreign-body giant cells were generally present, and macrophages persisted at the injection site for 3 months or more.

The area of the submucosa described at 7–10 days as showing a homogeneous eosinophilic appearance changed steadily with time. The tissue composing this area became more dense in appearance and stained rather diffusely, showing increasing eosinophilia (H and E) and fuchsinophilia (PAS). With PTAH the area stained a pale tan early, and later acquired an increasingly darker brown color. The staining reaction with VG changed from a pale orange through pink to red. No amyloid could be demonstrated. With time, a chronic inflammatory reaction characterized by focal collections of lymphocytes and plasma cells gradually made its appearance in the submucosa, well beyond the injection site.

In a few cases an ulcer extended into the injection site followed by evacuation of the crystalline and necrotic contents. In a few other cases a squamous-lined cystlike structure formed at the injection site, and persisted for months (fig. 27).

Changes in the mucosa: Acanthosis and hyperkeratosis of slight degree developed in the mucosa during the first 3½ days (fig. 12) following injection of methylcholanthrene. The cells of the basal layer became altered in their orientation and staining characteristics, and many of them appeared edematous and swollen. Individual, distorted cells containing elongated, hyperchromatic (pyknotic?) nuclei were interspersed between adjacent swollen basal cells. Immediately above the basal layer of cells

was a layer of compressed cells with deeply basophilic nuclei (fig. 12). Next above were the cells of the spinous layer, showing both an increase in the amount of cytoplasm and enlargement of the nuclei in which the chromatin was coarser and the nucleoli larger than normal. A few of the prickle cells contained multiple small eosinophilic granules (prekeratohyaline granules?) in their cytoplasm. There was a prominent granular layer with an occasional cell containing a rather large nucleus and with keratohyaline granules that varied in size. The number of mitotic figures did not appear to be increased.

At the end of the first week the acanthosis and hyperkeratosis were sufficiently marked to produce a plaque 4 mm. in diameter on the mucosal surface of the forestomach. The disturbance of differentiation and keratinizaton of the cells was more pronounced. Some of the keratinized cells retained distorted pyknotic or ghost nuclei. A few large, round eosinophilic bodies resembling corps ronds were enmeshed in the layers of keratin. The normal wavy outline marking the junction between the mucosa and the lamina propria became accentuated, so that it resembled more the junction between the rete pegs and the papillary layer of the epidermis (fig. 20). At the tip of a few of these retia-like projections the cells of the basal layer were absent (fig. 16). There was one abnormal mitotic figure. A single projection of epithelial cells extended into the muscularis mucosae, which was the first evidence of infiltration.

By the tenth day the mucosal plaque, slightly larger now, was composed of several layers of keratin attached to the acanthotic mucosa, which was seven to eight cells in thickness compared to a normal mucosa only three or four cells thick. There were focal areas of atypical parakeratosis and small, loosely laminated spherules of keratin, both in the acanthotic mucosa and in the overlying keratin. Mitotic figures in the cells of the basal layer were frequent. As in the 7-day specimens, the tip of a few of the retia showed focal loss of basal cells.

At 2 weeks the mucosal plaque contained a central stellate cleft (fig. 11) and, as previously, showed atypicality of cells and alteration of differentiation and keratinization. In one of the two specimens studied, the reticular basement membrane was disrupted around two separate strands of epithelial cells that had infiltrated the submucosa (fig. 24).

Two 17-day specimens showed infiltration of epithelial cells into the submucosa (figs. 25, 28, and 31). One of these specimens (fig. 28) exhibited a diverticulum-like lesion that did not quite reach the serosa. In the mucosa lining the tip of this diverticulum was a focus of leukocytes, above which the keratin was interrupted. Around the focus of leukocytes the squamous cells were loosely arranged and dyskeratotic, and below it the basal cells were absent (fig. 31).

The other 17-day specimen showed a significant lesion (noted previously at 7 days and subsequently in a number of the forestomachs) characterized by inversion of keratinization at the blunt tip of one or more retia (figs. 17, 18, and 19). Normally the stratified squamous epithelium of the

mucosa keratinizes only in the direction of the lumen of the forestomach; whereas its deep aspect, which is in contact with the lamina propria, is covered by basal cells. In the lesion characterized by inversion of keratinization, the deep aspect of the individual rete was covered, not by basal cells, but by keratin. There was, then, bilateral keratinization both toward the lumen of the forestomach and in the direction of the underlying lamina propria. Depending upon the plane of the section, these two areas of keratin were either continuous or apparently separated from each other by a zone of atypical epithelial cells, most of which appeared to be spinous in character. The sides of the involved rete were covered by basal cells down to the level of the plug of keratin, at which point they curled inward to become continuous with the spinous cells, which were differentiating into keratin in both directions. The connective tissue stroma in contact with the keratin showed no inflammatory or foreign-body reaction.

Dyskeratosis: From the 3d to the 17th day as described, and indeed throughout the subsequent weeks of the experiment, changes developed in the acanthotic and hyperkeratotic mucosa which, for want of a better term, have been designated dyskeratosis. This dyskeratosis was characterized by prematurely keratinized single cells, corps ronds, and atypical parakeratotic cells. The viable cells around the dyskeratotic foci frequently exhibited alteration in size, shape, and staining characteristics.

A hitherto unobserved feature of the disorder of keratinization was the occasional presence of stainable cytoplasmic granules in the epithelial cells of the different layers of the mucosa. These altered cells were seen at different stages of the experiment but were most conspicuous in a specimen of forestomach examined 35 days following injection of the methylcholanthrene (figs. 20 and 21). The granules in the altered cells in the lower spinous and basal layers were acidophilic in the H and E and PAS preparations, reddish brown with PTAH, greenish yellow with VG, and black following silver impregnation. In the silver preparations, these granules were not only black but otherwise were morphologically identical to the keratohyaline granules in the cells of the granular layer. In the stained preparations, a gradual transition in staining characteristics could be traced between the granules within the epithelial cells of the different layers of the mucosa and those of the granular layer. The conclusion seemed inescapable that some of the keratohyaline granules in the granular layer were transformations of these newly observed granules in the cells deep in the mucosa.

Proliferation of basal cells: Following initial edema and distortion of the basal layer at 3 days, and the degeneration and focal loss of the basal layer observed at 1 week and 10 days, focal proliferation of the basal cells of the mucosa developed. This basal-cell proliferation was apparent first in a specimen examined 17 days following injection, and was frequently observed thereafter. The basal cells became irregular in size, shape, staining characteristics, and orientation, and formed budlike processes which imparted a scalloped appearance to the sides and base of the in-

volved retia (fig. 23). These budlike processes impinged upon, distorted, and often penetrated the muscularis mucosae following disruption of the basement membrane (figs. 22 and 23).

The most extreme example of basal-cell proliferation manifested itself as a papillary growth in a forestomach specimen 162 days after injection (fig. 32). The growth was composed almost entirely of sheets and cords of elongated, sometimes spindle-shaped basal-like cells with hyperchromatic, ovoid, or elongated nuclei. Several spherical masses of keratin were present in the depth of this growth. The transition from tumor cells to keratin was abrupt. The lesion bore a superficial resemblance to the seborrheic keratosis of man, although the infiltration at the base of the experimental lesion suggested its malignant potentiality.

Infiltration: When the altered cells of the mucosa showed evidence of aggressively infiltrating the stroma, or when an inflammatory process spread from the site of injection to involve the undersurface of the mucosa, the reticular basement membrane coincidentally became disrupted (figs. 22 and 23). However, after the infiltrating neoplastic cells had extended more deeply into the gastric tissues, the collagen and reticulum became condensed about them and apparently formed a new stroma.

The first infiltrating lesion in which the neoplastic epithelial cells penetrated all coats of the forestomach and formed a tumor nodule on the serosa, was observed in an animal killed 60 days following injection of methylcholanthrene. By definition, therefore, this represents the earliest example of carcinoma. Atypical epithelial cells were, however, observed infiltrating the muscularis mucosae at 7 days and the submucosa in one specimen at 13 days, and in two specimens at 17 days. Moreover, neoplastic foci in several specimens observed at biweekly intervals between 17 and 60 days, and thereafter exhibited more aggressive infiltrating characteristics although falling short of fulfilling the criteria of carcinoma as defined. Along with penetration of the wall of the forestomach in depth, the neoplastic tissue extended laterally from its points of origin, infiltrating the wall of both chambers of the stomach and of the esophagus; following penetration of the serosa many of the surrounding organs and structures became infiltrated by neoplastic tissue.

DIVERTICULUM

In table 2, 22 lesions were classified as a diverticulum (group 1). Although the wall of the forestomach regularly showed a defect and the deep aspect of the diverticulum was sometimes adherent to the spleen, liver, and other extragastric structures, still the stratified squamous epithelium lining some of these lesions appeared so innocent that a diagnosis of carcinoma could not be entertained (fig. 29). By contrast, other examples of the diverticulum exhibited neoplastic proliferation of the lining mucosa, with infiltration of the adjacent stroma and muscularis to a degree that made it difficult to distinguish these lesions from carcinoma. Still other diverticular lesions were associated with genuine carcinoma (fig. 30), and these specimens are included in group 2 of table 2. Some diverticular

lesions, when small and shallow, were distinguished with difficulty from the umbilicate lesion that develops in the forestomach of mice ingesting methylcholanthrene (*6*), and that so frequently eventuates in carcinoma. At the growing tip of many of the diverticula there were alterations of dyskeratosis in the epithelial cells as described at 17 days (fig. 31).

CONTROL MICE

No lesion of any type was found in the forestomach of the 15 control animals. In most of the specimens the exact site of injection could not be identified with certainty.

DISCUSSION

A single dose of 0.6 mg. of methylcholanthrene injected into the submucosa of the forestomach of mice, killed at varying intervals thereafter, induced squamous-cell carcinoma, sarcoma, mixtures of these growths, and precancerous lesions; the latter are believed to be of importance in the evolution of the malignant neoplastic process.

The term "precancer" has been used in clinical and experimental medicine to designate a group of lesions or conditions that are early cancers or cancers with or without limited infiltration or that are followed more or less consistently by cancer. Some of these are almost always followed by cancer sooner or later; others frequently give rise to cancer, while in still others cancer occurs only occasionally within the limits of the life span of the animal. In the present experiment, the lesions listed as precancerous in table 2 include all the forestomachs not showing carcinoma by definition. Many of the obviously neoplastic lesions showed infiltration of varying extent, sometimes just short of penetration of the serosa which, in this experiment, is the dividing line for distinguishing between precancerous lesions and carcinoma. Other lesions classified as precancerous were those occurring early in the experiment. The basis for considering these latter lesions as precancerous is validated by the high incidence of overt carcinoma developing under the conditions of this experiment.

The question as to when an induced neoplastic lesion of the forestomach warrants a diagnosis of carcinoma, constantly disturbs the investigator working in the field of experimental gastrointestinal cancer. In this as in previous reports from this laboratory carcinoma has been defined as a neoplastic lesion that infiltrates all coats of the viscus and forms a tumor mass on the serosa. For several reasons these criteria are not entirely satisfactory. On this basis some of the precancerous lesions in the present experiment fell just short of being classified as carcinoma. Some of the diverticula, although they extended through the serosa and also frequently exhibited neoplastic infiltration of the wall of the forestomach laterally, did not show neoplastic infiltration through the serosa. An attempt was made to diagnose carcinoma of the forestomach by the same general criteria employed in diagnosing gastric carcinoma in man, where the welfare of the patient is of paramount importance. Several pathologists familiar with the lesions of gastric carcinoma in man and in the experimental animal, and others less familiar with the experimental lesion,

were asked their opinion of a number of debatable lesions. The opinions were divergent; the tendency of the latter group was to classify more of the experimental lesions as carcinoma rather than as precancerous. Furthermore, a given pathologist's opinion varied from time to time. For example, one pathologist after examining the lesions listed under the heading of diverticulum, classified several of these as carcinoma. A few months later the same pathologist reviewing the same material classified them as diverticula. This points up a real dilemma. From the standpoint of interpretation of experimental results, the criteria employed are rigid and perhaps ultraconservative; however, they provide a margin of safety in arriving at conclusions from experimental work (*8*). From the standpoint of diagnosing human gastric cancer, adherence to such rigid criteria would be thoroughly unsound and misleading, for in this realm knowledge is more extensive and the purpose of the examination is different. It might be argued that infiltration of atypical cells, even in minor degree, is an adequate criterion of experimental cancer. If, however, this criterion is accepted, then it would be necessary to classify as carcinoma one lesion of the forestomach at 7 days, another at 13, two at 17 and several others prior to 60 days, postinjection, for all these specimens showed atypical epithelial cells infiltrating not only the lamina propria but beyond it. Infiltration of this degree may be observed in the forestomach of mice or rats subjected to a variety of experimental conditions such as starvation and vitamin deficiency, which do not evoke cancer. It might be argued further that no good purpose is served by making a distinction between precancerous lesions and carcinoma. Certainly in view of the high incidence of lesions of infiltrating and metastasizing carcinoma in the present experiment, the majority of the associated changes described as precancerous are in all probability carcinoma in miniature.

Seventy-one percent of the precancerous lesions in the present groups of mice were observed during the first 16 weeks of the experiment, and 29 percent in the succeeding weeks. In both periods similar lesions were observed. This lends support to the belief that many of the lesions observed in the later period were also potentially malignant although the tempo of the neoplastic process was less rapid. Many of the changes in the forestomach interpreted as precancerous in the present experiment were also observed in a previous experiment in which methylcholanthrene was administered orally to mice (*6*). The present experiment is complicated by the puncture wound of the forestomach at operation and by the deposit of methylcholanthrene in the submucosa, with the attendant severe local inflammatory and degenerative changes. The gastric wall was weakened following destruction of the supporting tissues and this, it is believed, led to the development of the diverticular lesion, which was not observed in the feeding experiments. Although some or all coats of the wall of the forestomach showed a defect, and the deepest aspect of the diverticulum was sometimes adherent to spleen, liver, and other extragastric structures, the stratified squamous epithelium lining some of

the lesions appeared so innocent that a diagnosis of carcinoma could not be entertained. There seemed to be as much justification for separating this diverticulum from experimental carcinoma as for distinguishing between carcinoma and chronic diverticulitis in man. In some instances, the epithelial lining at the deepest extension of the diverticulum showed dyskeratosis and loss of basal cells. What role, if any, these dyskeratotic cells played in accelerating the deep extension of the diverticular lesion is problematical, but there was abundant evidence that precancerous changes and carcinoma developed. The diverticulum may have been preceded in some instances by the umbilicate lesion that occurred so frequently in the feeding experiments but was only occasionally observed in the injection experiment. In the feeding experiments localized damage to the supporting tissues of the forestomach was less severe, and this probably accounts for the failure of the umbilicate lesions to form diverticula.

The role of the degenerative changes in the stroma, muscularis, and walls of the blood vessels in the histogenesis of carcinoma in the injection experiments is probably similar to that of the analogous but less severe changes in the feeding experiments. The importance of these in the pathogenesis of carcinoma developing in the epidermis of the skin has been the subject of a great deal of discussion and speculation since the early studies of cutaneous carcinogenesis by tar and later by the hydrocarbons. In the present experiment an unusual lesion of undetermined nature was observed in the connective tissue of the submucosa adjacent to, and for some little distance beyond, the focus of methylcholanthrene. The involved tissue appeared edematous, but if this change was due to edema, then the edema fluid must have had a high content of a stainable material. With time, the areas of involvement became more dense in appearance and showed increasing eosinophilia and moderate fuchsinophilia. The connective-tissue stains suggested an increase in collagen. The nature of this change has not been fully elucidated.

Lesions characterized by acanthosis, hyperkeratosis, basal cell proliferation, and malignant dyskeratosis of the epithelium were observed in many specimens throughout the course of the present experiment and in many particulars were like these respective changes in the feeding experiments. A distinctive difference, however, was observed in the injection experiment. This was characterized by the appearance of prekeratohyaline granules in the cells of the basal and lower spinous layers. These granular objects were blackened in the preparations impregnated with silver, just as were the keratohyaline granules in the granular layer. With the staining procedures employed, although the granules at the two extremes of the width of the epithelium stained differently, those in cells in between showed transitions in staining characteristics. This, coupled with the fact that morphologically the granules in the cells of the basal layer and the keratohyaline granules in the granular layer were indistinguishable, lends credence to the belief that at least some of the latter were derived from the former.

The lesion that occurred at the blunt tip of one or more of the retia and that has been designated inversion of keratinization was not observed in the feeding experiments. This lesion occurred in specimens obtained at 7 and 17 days, and in a number of others subsequently. It was associated with loss of the basal layer of cells covering the tip of the rete and loss of the reticular basement membrane. This lesion showed keratinization taking place in two directions—toward the lumen of the forestomach, as normally, and toward the underlying stroma. The keratin was formed by spinous cells remaining after loss of the cells of the basal layer. The pathogenesis of this lesion, although imperfectly understood, would appear to be of significance as a possible early stage in the mechanism of infiltration.

SUMMARY AND CONCLUSIONS

A single dose of 0.6 mg. of methylcholanthrene in a concentrated aqueous methocel suspension was injected intramurally into the submucosa of the forestomach of strain C57 brown and A backcross hybrid mice. The experiment extended over a period of 77 weeks, during which time 99 of 165 treated animals developed a malignant tumor. Many animals were killed at intervals, according to a prearranged schedule, to determine the latent period of carcinoma and to afford material for a study of the histopathogenesis of forestomach carcinoma.

Of 132 animals autopsied following the latent period (after 60 days), 99 had a malignant tumor of the forestomach; of these 83 were squamous-cell carcinoma, 4 sarcoma, and 12 mixtures of squamous-cell carcinoma and sarcoma.

The complicated and often interrelated morphologic changes occurring in the squamous mucosa, stroma, blood vessels, and muscularis of the forestomach during the evolution of the malignant neoplastic process are described and illustrated and are compared and contrasted with those induced by oral administration of the carcinogenic hydrocarbons. The observations described emphasize the complexity of the process of experimental carcinoma and controvert the frequently expressed opinion that the mechanism of carcinogenesis resides in a single cell.

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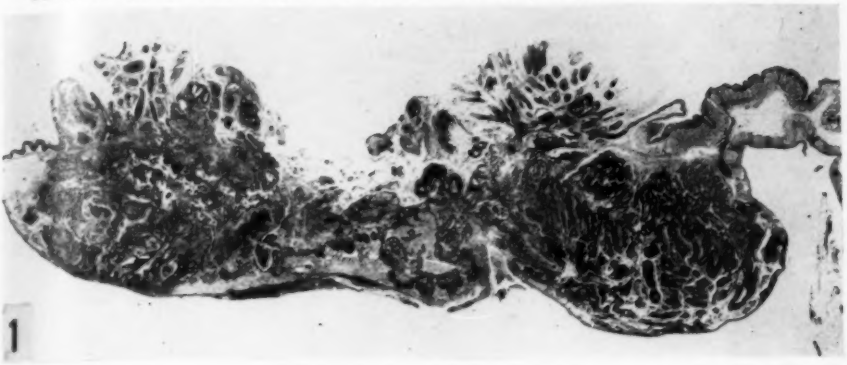
PLATE 43

The illustrations of the forestomach are all taken from the region of the injection site. The histologic sections illustrated are stained with hematoxylin and eosin unless otherwise stated. The period following injection is given in days. All the carcinomas are of the squamous-cell type, and all tumors are primary in the forestomach.

FIGURE 1.—Forestomach—carcinoma. 147 days. × 16.
FIGURE 2.—Abdominal peritoneum—implant of carcinoma. 196 days. × 120.
FIGURE 3.—Regional lymph node—metastatic carcinoma. 118 days. × 150.



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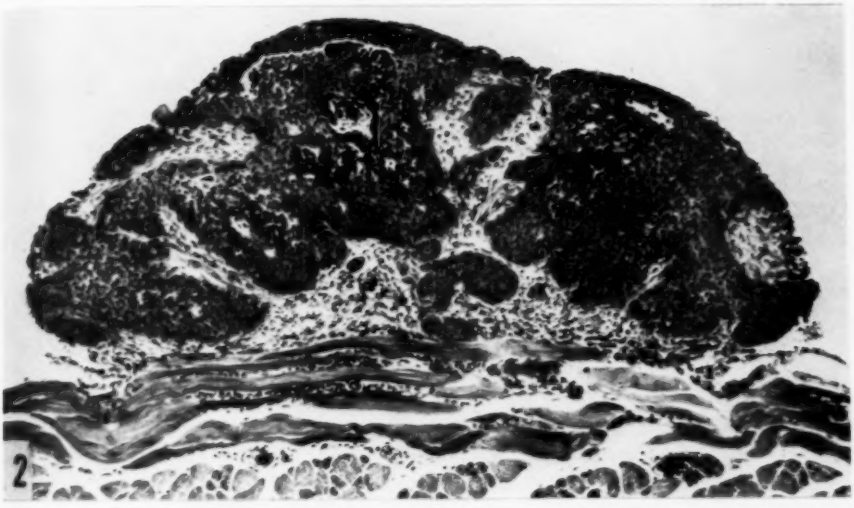

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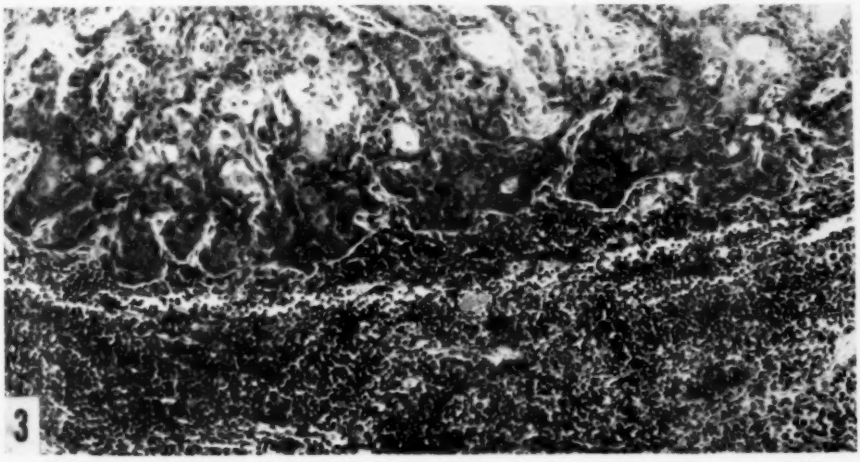

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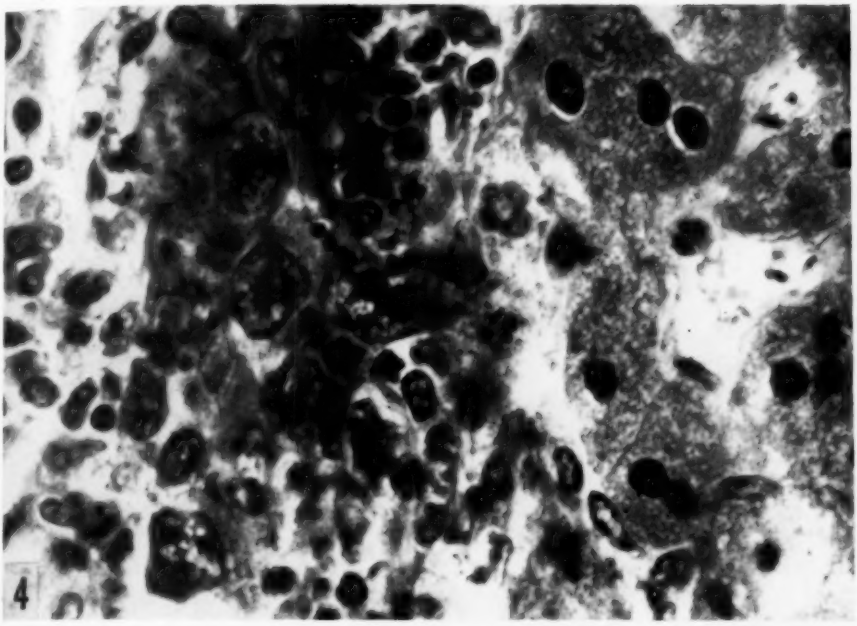
PLATE 44

FIGURE 4.—Liver—focus of atypical cells, probably metastatic carcinoma. 91 days. × 1,120.

FIGURE 5.—Liver—metastatic carcinoma. 118 days. × 360.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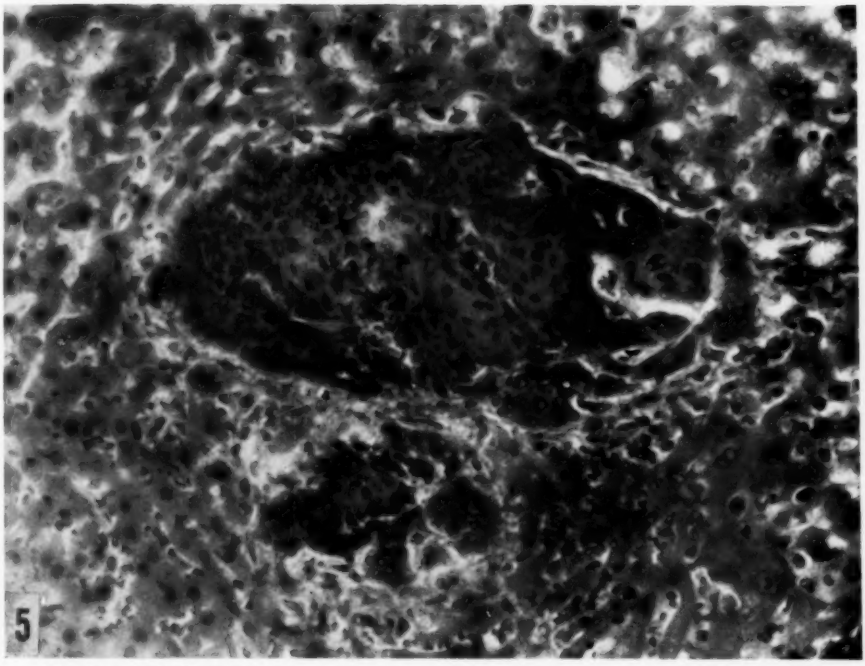
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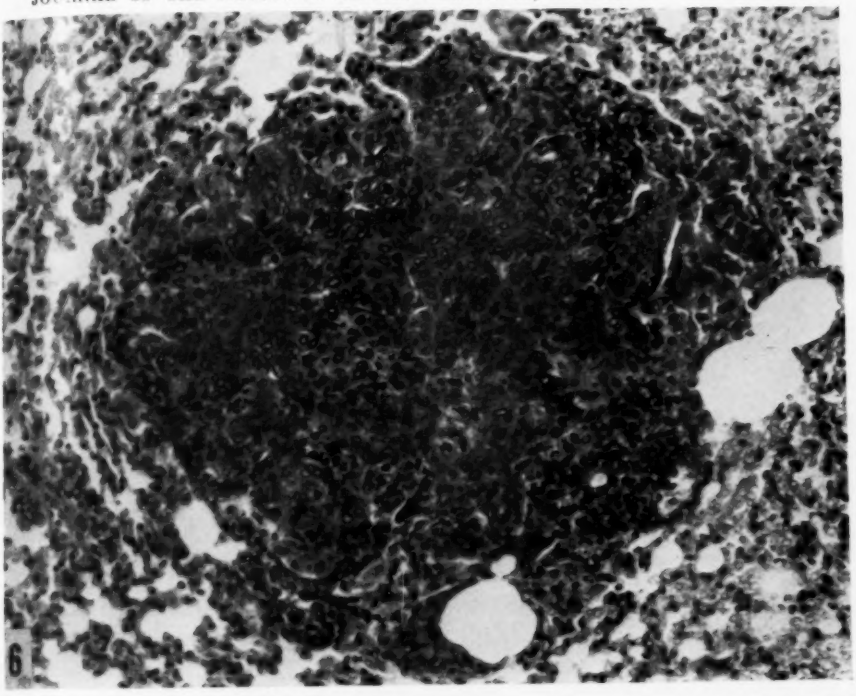
PLATE 45

FIGURE 6.—Lung—metastatic carcinoma. 196 days. × 360.
FIGURE 7.—Forestomach—mixed carcinoma and sarcoma. 151 days. × 95.
FIGURE 8.—Forestomach—sarcoma. 161 days. × 85.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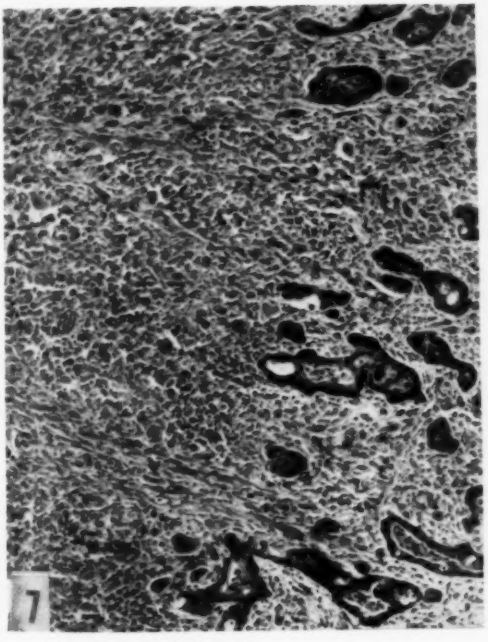
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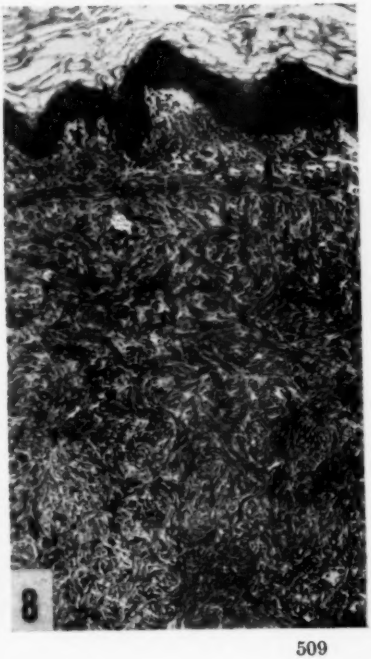
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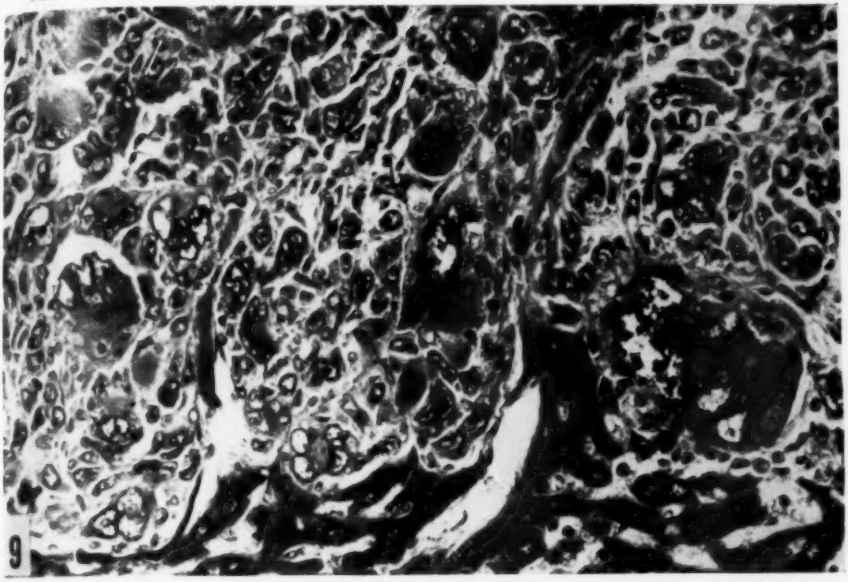
PLATE 46

FIGURE 9. Liver—infiltrating sarcoma with bizarre tumor giant cells. 133 days. × 340.

FIGURE 10.—Wall of forestomach—bundles of atypical spindle cells, possibly presarcoma. 56 days. × 285.

FIGURE 11.—Esophagus, stomach, and duodenum—hyperkeratotic nodule of forestomach just proximal to limiting ridge overlying site of injection. 13 days. Photograph of gross specimen. Circa × 4.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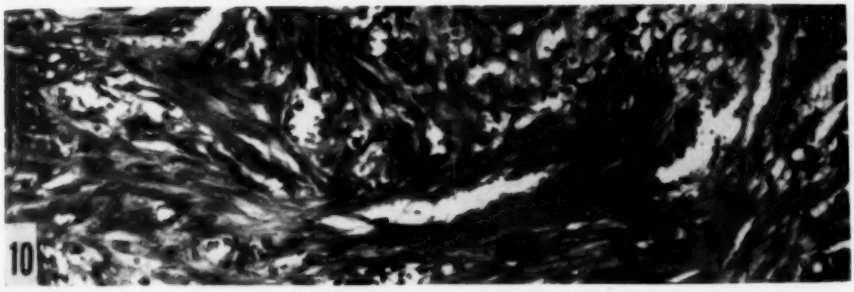
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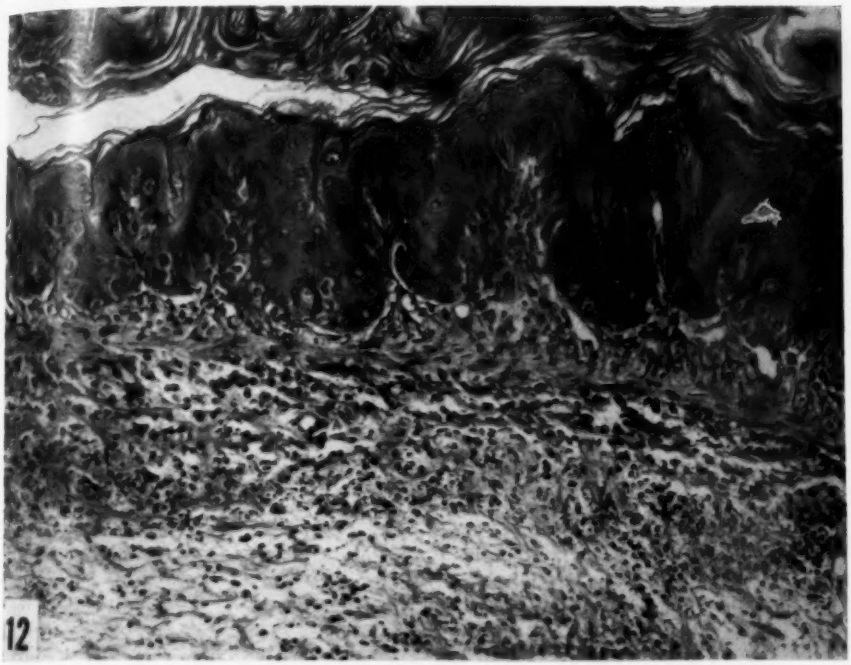
PLATE 47

FIGURE 12.—Forestomach degeneration and necrosis at injection site. Above this there is an infiltration of polymorphonuclear leukocytes in the submucosa, muscularis mucosae, and lamina propria. There is retia-like accentuation of the mucosal folds, which show cellular crowding of basal layer with variation in size and staining of nuclei and edema of cytoplasm. 3 days. × 180.

FIGURE 13.—Forestomach—injection site with liquefaction centrally and degeneration of collagen at the periphery. 17 days. PTAH. × 95.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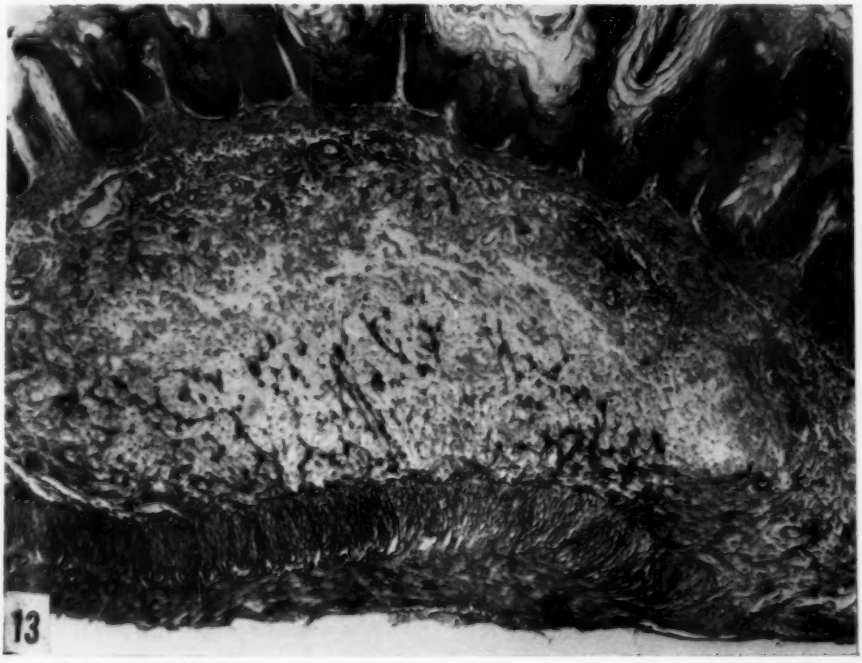
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PLATE 48

FIGURE 14.—Forestomach—the injection site contains necrotic exudate and crystal clefts, some within macrophages. There are dilatation of capillaries and a smudged appearance of the submucosa. 21 days. × 95.

FIGURE 15.—Forestomach—to the left are vacuolated macrophages and other inflammatory cells bordering injection site. To the right the connective tissue is pale and smudged. There is degeneration of the muscularis propria. 31 days. × 140.

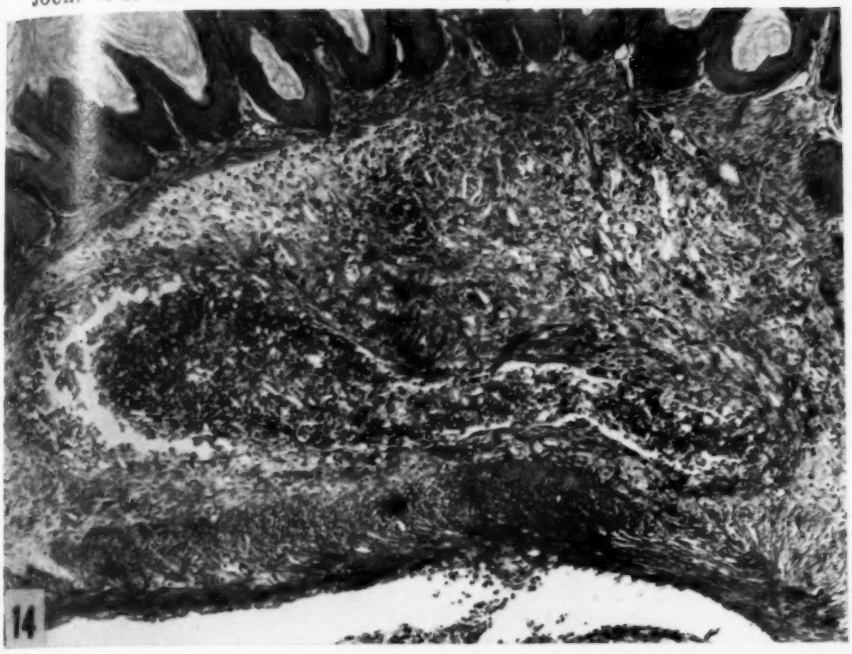
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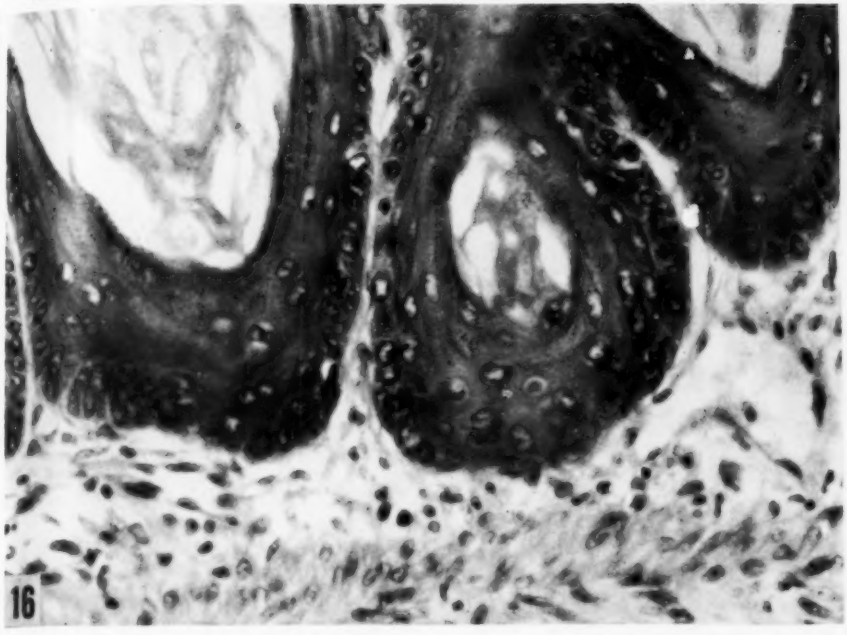
PLATE 49

FIGURE 16.—Forestomach—there are cellular variation in the mucosa and focal loss of basal cells. 10 days. × 690.

FIGURE 17.—Forestomach—a mucosal rete shows inversion of keratinization; plane of section is off-center (compare with that of two of the retia in figure 18) accounting for the bridge of cells interposed between the apparently separated masses of keratin above and below. 7 days. × 690.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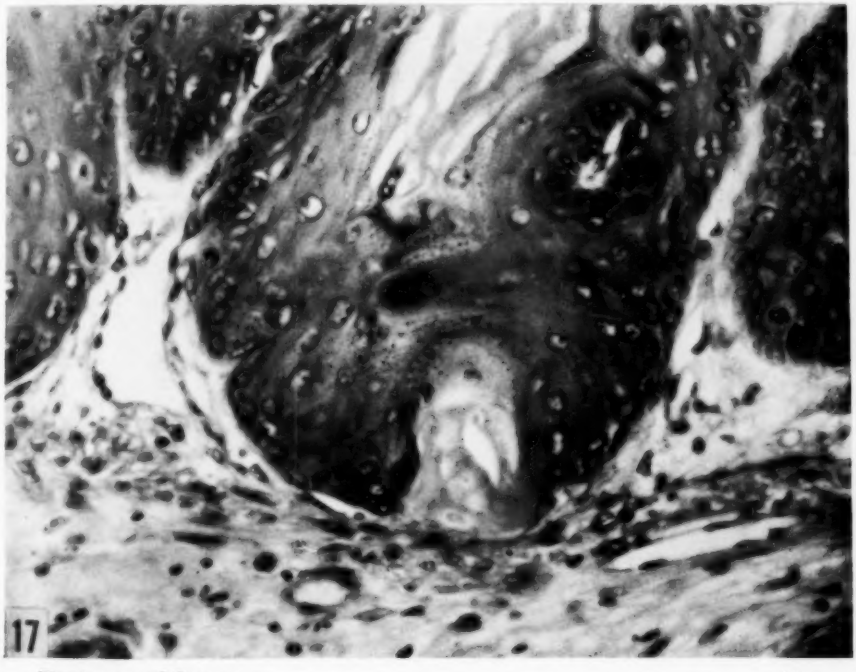
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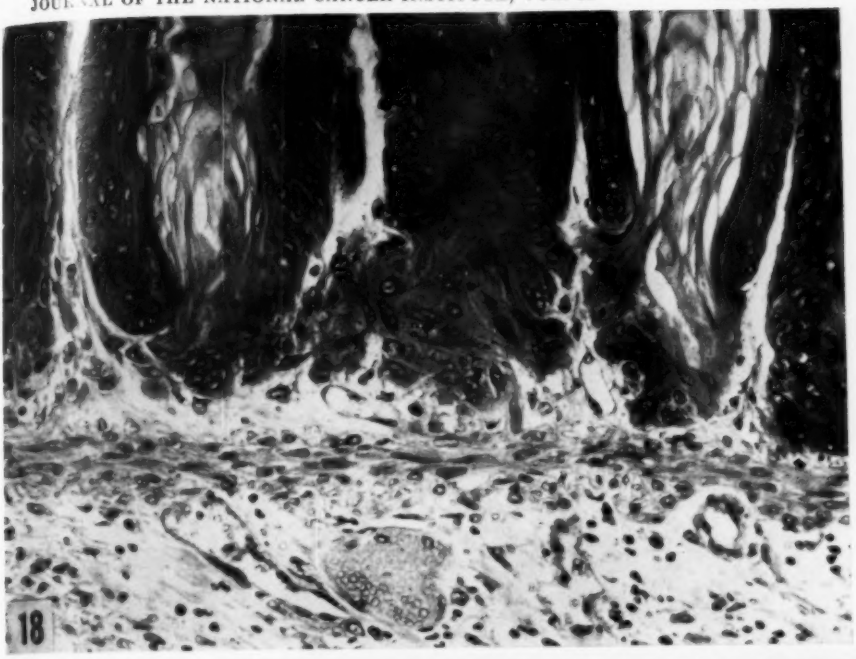
PLATE 50

FIGURES 18 and 19.—Forestomach—several mucosal retia showing loss of the basal layer with keratin and spinous cells in direct contact with the lamina propria. This appears to be a beginning stage of invasion of lamina propria. 17 days. × 300.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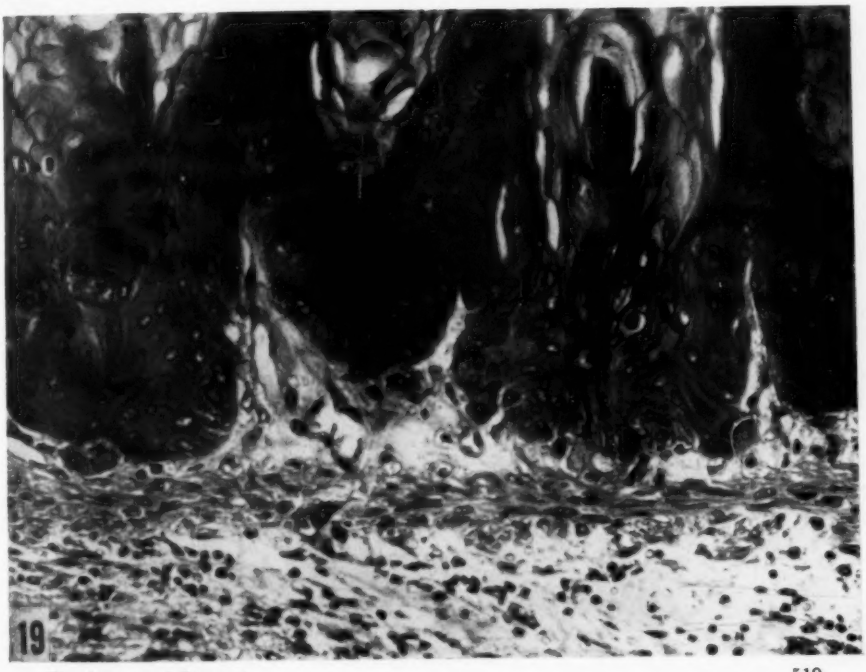
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PLATE 51

FIGURE 20.—Forestomach—argyrophilic cytoplasmic granules in basal and spinous cells considered to be prekeratohyaline granules. 35 days. Wilder's silver impregnation for reticulum. × 415.

FIGURE 21.—Higher power of area adjacent to that in figure 20, showing argyrophilic granules. Wilder's silver impregnation for reticulum. 35 days. × 1,400.

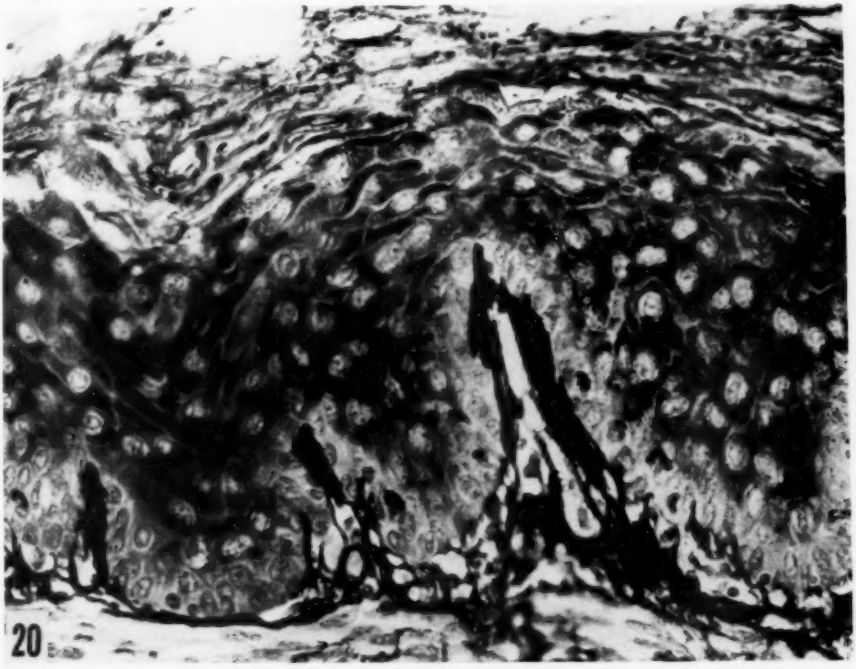

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PLATE 52

FIGURE 22.—Forestomach—interruption and fragmentation of reticular basement membrane. Argyrophilic fibrils are interspersed in the keratin. 17 days. Wilder's silver impregnation for reticulum. × 300.

FIGURE 23.—Forestomach—penetration of reticular basement membrane by tongues of basal cells. 40 days. Wilder's silver impregnation for reticulum. × 300.

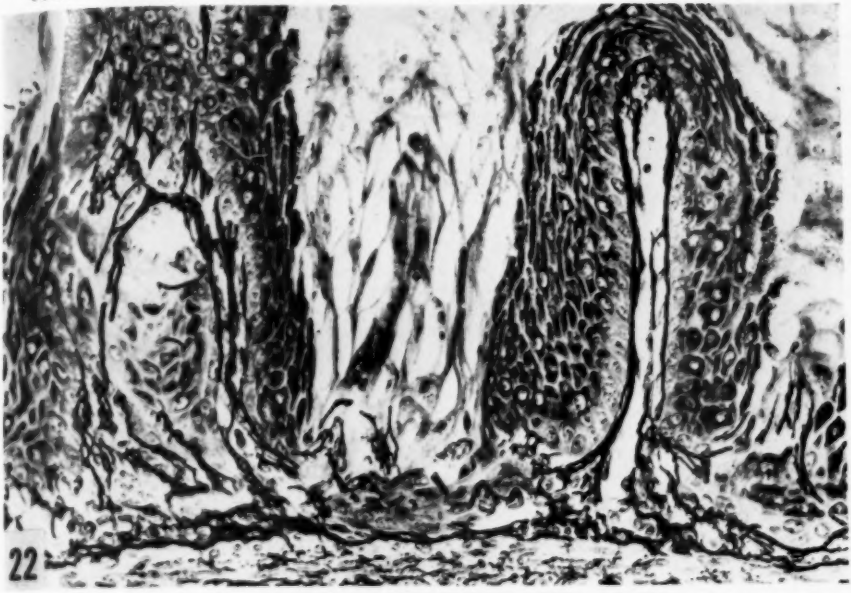
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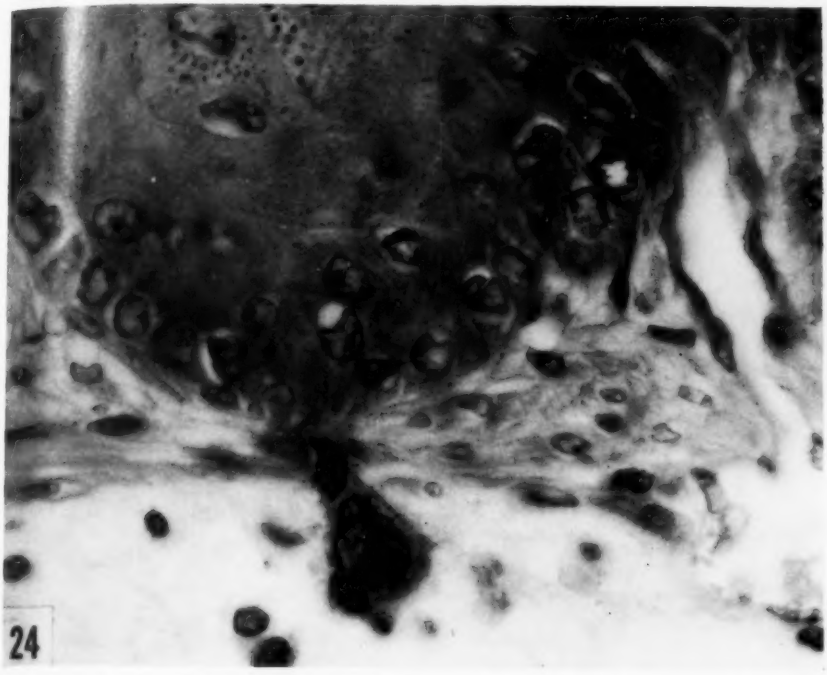
PLATE 53

FIGURE 24.—Forestomach—budlike process of the mucosa has penetrated the muscularis mucosae and extends into submucosa. 13 days. × 1,340.

FIGURE 25.—Forestomach—the muscularis is necrotic and smudged. It is infiltrated by epithelial cells, which extend into the submucosa. 17 days. × 300.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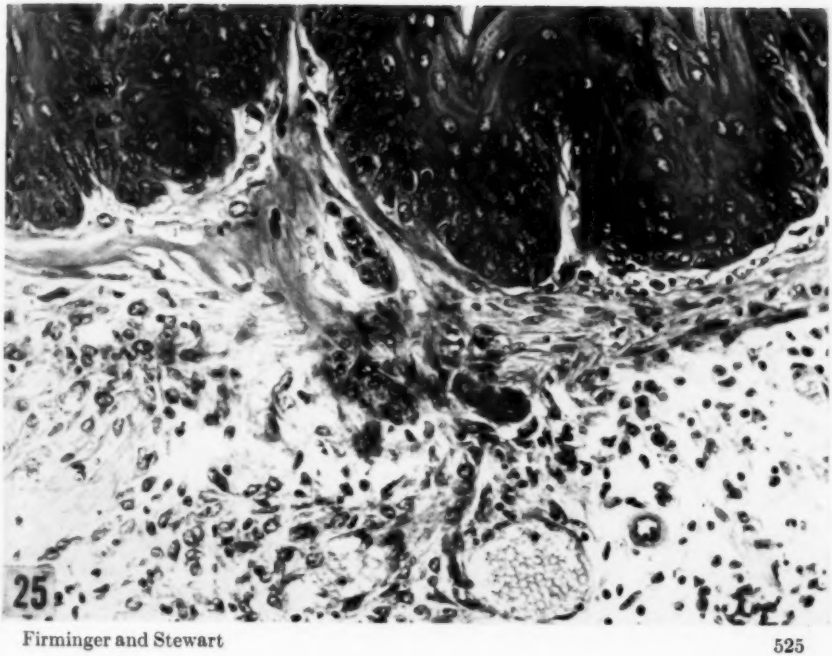
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PLATE 54

FIGURE 26.—Forestomach showing acanthosis and hyperkeratosis of the mucosa with neoplastic invasion of the submucosa, which has a hyaline appearance. 56 days. × 38.

FIGURE 27.—Forestomach showing broad umbilicate lesion, which has extended through the muscularis propria but is confined by the thickened, chronically inflamed serosa. There are squamous-lined cystic structures on each side. 163 days. × 38.

FIGURE 28.—Forestomach—a diverticulum extends into the subserosa, which is thickened by fibrous inflammatory tissue. Much of the muscularis propria is destroyed in this area. Tip of diverticulum is shown in figure 31. 17 days. × 39.

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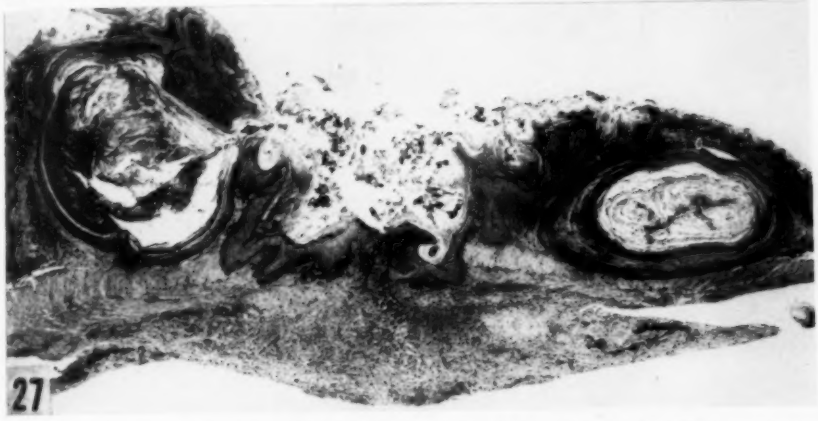
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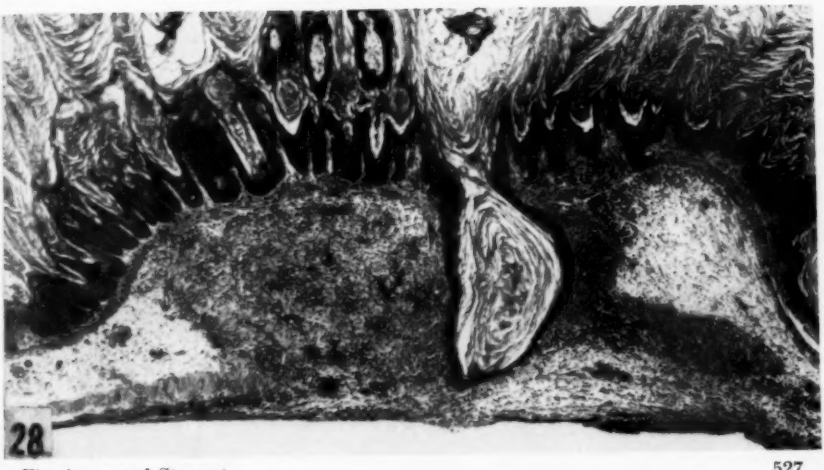
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PLATE 55

FIGURE 29.—Forestomach—large diverticular lesion with bulbous distention on the serosa. In all probability this lesion is in part the result of herniation of the mucosa due to damage to the supporting structures of the wall of the forestomach. 26 days. × 42.

FIGURE 30.—Stomach, pancreas, and spleen—a diverticular lesion extends beyond the stomach and is attached to the spleen. There is a large carcinoma involving the neck and wall of the diverticulum and the wall of both chambers of the viscus. 141 days. × 15.

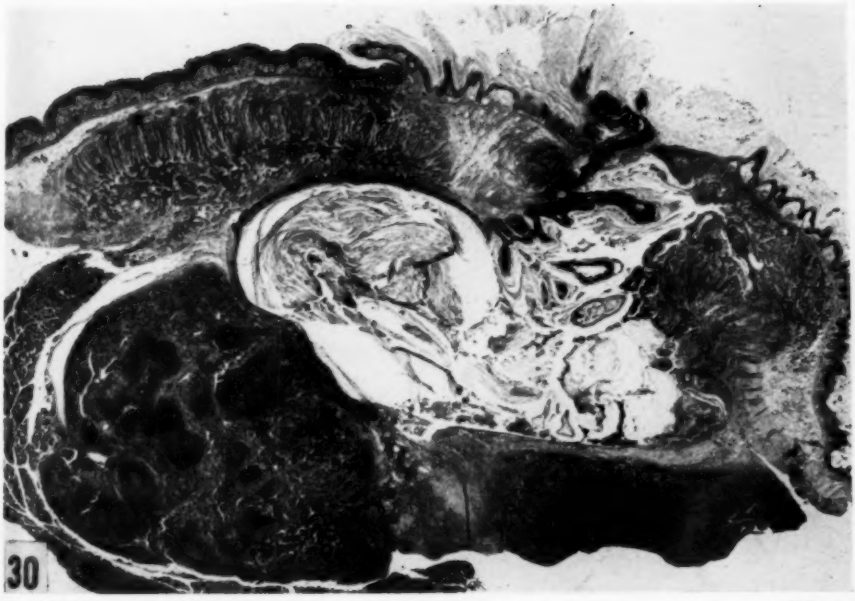

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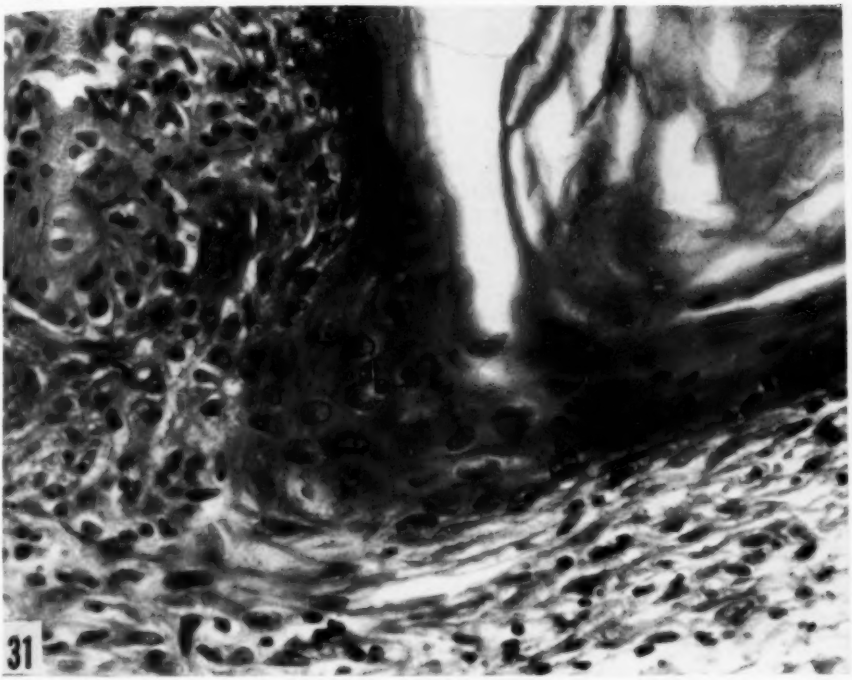
PLATE 56

FIGURE 31.—Forestomach—tip of diverticulum illustrated in figure 28 showing loss of basal layer and irregularity of epithelial cells. × 690.

FIGURE 32.—Forestomach showing papilloma composed of atypical basal cells and somewhat resembling a seborrheic keratosis. There are invasion of the submucosa and impingement on the muscularis propria. 162 days. × 85.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THE 30-DAY LD-50 OF TWO RADIATIONS OF DIFFERENT ION DENSITY [1]

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INTRODUCTION

Ever since the development of accelerators for the production of X and corpuscular radiation in the million and multimillion volt range, the effect of radiation quality on biologic response has become increasingly the subject of much discussion and investigation. Most of these investigations have dealt with small biologic entities such as bean and wheat roots, viruses, bacteria, spores and in-vitro studies on tumor tissue. Few in-vivo studies on tumors or whole mammals have been made. Tobias (*1*) found that the LD–50 for mice was the same for X-rays as for 200-Mev deuterons. This is not surprising, as the specific ionization density is approximately the same. However, differences might be found for radiation of great differences in specific ionizations. Hagen and Zirkle (*2*) showed that the efficiency of neutrons with a maximum energy of 12 Mev was four times that of X-rays as measured by the LD–50 in mice and 2.5 as measured by the LD–50 in rabbits.

Wherever absorption of radiation takes place, the resulting emission of electrons forms ions along its path. These ions may undergo chemical changes. When the greater part of the ionizations occur in water, as in biologic matter, OH and H radicals are formed that react with the cellular constituents. This fundamental ionization process is characteristic of all ionizing radiations, whether corpuscular or electromagnetic. A qualitative difference in the biologic effect of the different types of radiations cannot be expected. However, a quantitative difference caused by the different specific ionizations of the various radiations can be expected—*e. g.*, in equivalent amounts of radiation, *i. e.*, absorbed energy, 80 ions per micron, for 200 kv X-radiation may produce a different quantitative effect than 3,700 ions per micron for the α particle from radon. When a single ionization is sufficient to kill a cell or organism, X-radiation will be more efficient than α-radiation (*3*). Thus in the inactivation of viruses, enzymes, and bacteria, X-radiation has been found to be as much as 20 times as efficient as α-radiation (*3*). Whereas, when a number of ionizing events are necessary to produce a change, the densely ionizing particles are more efficient as

[1] Received for publication September 20, 1951.

[2] National Institutes of Health, Public Health Service, Federal Security Agency.

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seen in the detailed study of chromosome changes in *Tradescantia* or the lethal effect on bean root tips (*4*).

Furthermore, a change produced by a single ionization event would be expected to be independent of the dose rate. On the other hand, a change produced by several ionization events should be dependent on the dose rate for X-radiation (if no recovery occurs), and also for α-radiation when the number of ionizations per particle is less than that required to produce the change.

In-vitro studies on destruction of tumor tissue show that X-radiation is 50 percent more effective than γ-radiation (*5*) and that neutrons (ion density about 300 ions per micron) are 2 to 3 times more effective than X-radiation (*6*). Moreover, neutrons with 1,500 ions per micron were found to be 24 times more effective than γ-radiation in destroying tumor tissue *in vivo*, and 9 times more effective *in vitro* (*7*). This may indicate a response of the animal body to the irradiation, which increases with ion density.

Hence, the relative effectiveness of acute irradiation by X- and α-radiation in killing the whole animal was investigated. These experiments and results are presented in Experiment 1. The α-radiation from RaA and RaC′ in a solution of radon in equilibrium, with its short-lived decay products RaA, RaB, RaC, and RaC′, was employed because it was readily obtainable. Not only is the ion density of α particles considerable, but nonuniformity of the irradiation dose is to be expected in tissues in which the radioactive materials concentrated. Therefore, these products were traced in the mouse, and the findings are reported in Experiment 2.

EXPERIMENT 1

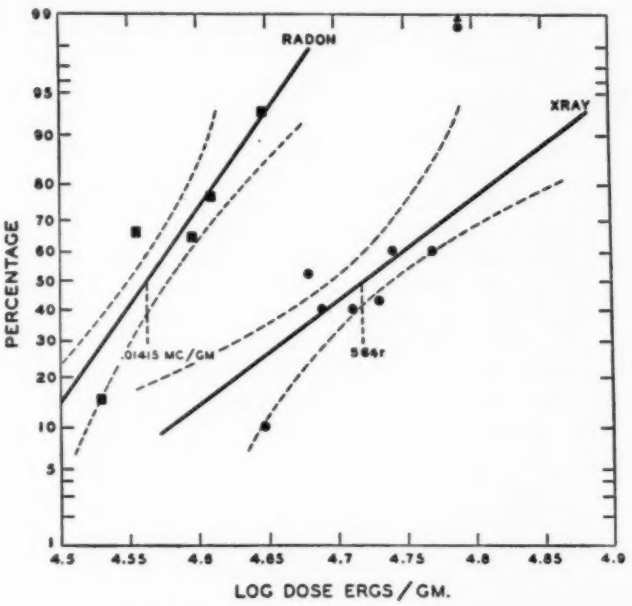
Method

Five groups of strain A male mice (Bittner subline), from 3 to 4 months of age, were injected intravenously with an aqueous solution of radon, in equilibrium with its decay products, containing 0.1 percent sodium citrate. The doses ranged from 0.013 mc./gm. to 0.017 mc./gm. or, expressed in energy absorbed mainly from the α particles of RaC′, 33,800 ergs/gm. to 44,200 ergs/gm. (*8*). From the percentage of animals in each group that died in 30 days the 30-day LD-50 was computed.

Eight groups of mice of the same age and strain were exposed to doses of X-radiation ranging from 480 to 645 roentgen, or 44,600 ergs/gm. to 60,000 ergs/gm. The X-radiation dose, which was given with a tube current of 20 milliamps and a tube voltage of 186 kvp. at a distance of 164 cm., extended over a 2-hour period. The dose rate was graduated by addition of aluminum and copper filters to simulate the dose rate of the radioactive material injected intravenously. That is to say, 16 percent of the dose was given in the first 10 minutes, 50 percent in the next 50 minutes, and 34 percent in the last hour. From the data obtained, the 30-day LD-50 was calculated for the two radiations.

Results

The percentage mortality increases rapidly with dose in the case of the radon-treated animals, but more slowly in the X-irradiated animals, as seen in table 1, where the findings are summarized, and in text-figure 1, which shows the log-probit regression curves. The X-radiation 30-day LD-50 as given here was 564 r, with a standard deviation of ± 23 r, or 52,400 ergs/gm., and for the radon solution 0.01415 ± 0.001 mc./gm., or 36,800 ergs/gm. The X-radiation energy absorbed is then 1.42 times that



TEXT-FIGURE 1.—Log-probit regression curves.

TABLE 1.—*Summary of 30-day LD-50 experiment*

Number of animals	Dose	Dose erg/gm.	Percent dead at 30 days
20	480 r	44, 000	10
40	515 r	47, 400	52
20	525 r	47, 900	40
20	567 r	52, 700	40
30	579 r	53, 800	43
20	593 r	55, 200	60
50	630 r	58, 600	60
17	645 r	60, 000	100
43	0.013 mc./gm	33, 800	15
38	0.014 mc./gm	36, 400	66
20	0.015 mc./gm	39, 000	65
34	0.016 mc./gm	41, 700	77
14	0.017 mc./gm	44, 200	93

of the α particle energy absorbed, if the LD-50's are compared. The variation of this ratio from 1.28 at 20 percent to 1.57 at 80 percent mortality is not great. The difference in slope is significant on the 95-percent level.

EXPERIMENT 2

Method

The radiation tissue dose from the decay products of radon cannot be expected to be evenly distributed throughout the animal as is the X-radiation tissue dose. The distribution of RaA, RaB, and RaC (and RaC') was followed for 2 hours after injection, at which time the total activity is negligible. As radon itself contributes only 2 percent of the total dose (*8*), and as the rapid diffusion of this gas makes accurate detection in any extracted tissue difficult, it was neglected. RaA and RaB also contribute little to the total dose, but the possibility exists of their concentration in one tissue and their production of a high dose in that tissue.

An aqueous solution (containing 0.1 percent sodium citrate) of radon in equilibrium with its decay products was injected intravenously into strain A male mice about 3 months of age. Each received 0.25 cc. containing from about 180 to 250 microcuries. Approximately 300 mice were used in the experiment, and three to nine mice were killed at specific time intervals. The tissue to be examined was excised, weighed on a torsion balance, and placed in a glass ampule. This ampule was then placed in a lead shield 3 mm. thick and the γ rays from RaC were counted with a Geiger counter connected to a count-rate meter. The radioactive decay of the sample was followed for 20 minutes and compared with radium standards, which consisted of ampules containing radium chloride solutions. Blood samples of 0.25 to 0.75 cc. were taken by heart puncture and were measured in the syringe. These samples were not taken at set intervals but at various times from 1 to 120 minutes after injection, because blood could not always be obtained on the first puncture.

From the decay of the sample, by applying the method of least squares to the formula for decay of RaC, the amount of RaC and RaA + RaB was calculated. As the first measurement could rarely be made in less than 2 minutes following the death of the animal, and as the initial amount of RaA, which has a 3-minute half life, is small, the amount of RaA was not obtainable. Hence, the sum of RaA and RaB was calculated. For dose calculations it was assumed that RaA was distributed through the body in the same manner as RaB; then the proportion $\left(\frac{\text{RaA}}{\text{RaA} + \text{RaB}}\right)$ would be 0.068 at 2 minutes, 0.037 at 5 minutes, and 0.005 at 15 minutes. For the most part, in the following pages this sum of RaA and RaB will be spoken of as RaB alone.

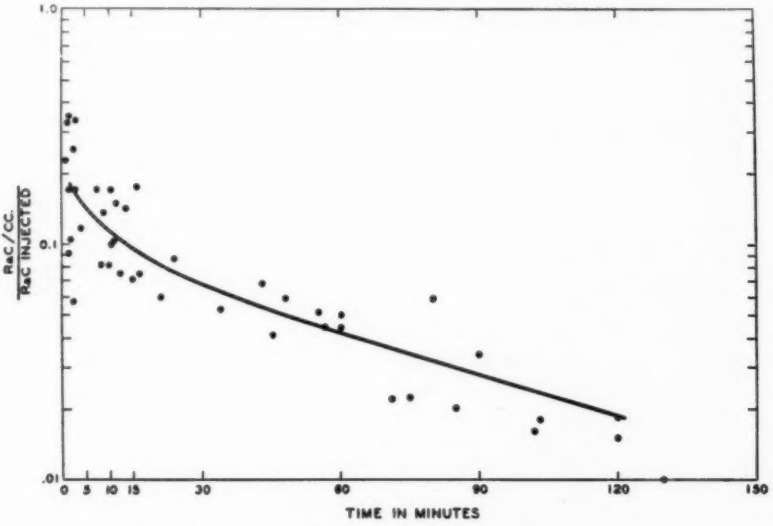
The dose was calculated for each tissue from the determined radioactive content by the method described by Cornfield (*9*). These doses were compared to the average dose or the expected X-radiation dose. In addition, the proportionate tissue weights were found and used to

calculate the total radioactive content of the tissues and the total energy absorbed in a 25-gm. mouse.

Results

The expression of accumulation and elimination of radioactive material in a biologic sample is of the form $N=\Sigma a_t e^{-(\lambda+b_t)t}$ where λ is the radioactive decay constant of the product, and a_t and b_t are constants. Since in this case a chain of radioactive products was involved, each b_t was a sum of the biologic decay constant and a radioactive decay constant.

For A, B, and C in the blood, this equation was found (text-figs. 2 and 3) to be: $N\,(A+B)=0.100e^{-0.047t}+0.0506e^{-0.0163t}$
$N\,(C)=0.117e^{-0.167t}+0.106e^{-0.0150t}$.[3] In each case the first term represents

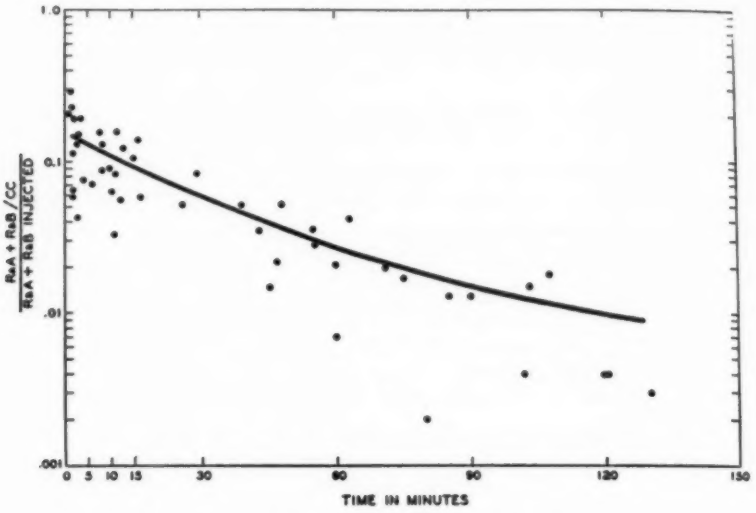


Text-figure 2.—RaC content of the blood.

the initial rapid pouring out into the tissues, while the second term, which becomes effective after 45 minutes, shows that the material is leaving the tissues and returning to the blood. This is seen when the radioactive decay constants of A, B, and C are compared with the values for b_t. The biologic half life of RaB in the blood from this time on is 43 minutes, while actually RaB has a half life of 27 minutes. Similarly, the biologic half life of RaC in the blood from 45 minutes to 2 hours after injection is 46 minutes, while RaC has a half life of 19 minutes and RaC in a solution of RaA, RaB, and RaC at equilibrium at 0 time has an effective half life of about 40 minutes during this period. No estimate of the amount in the blood has been made before 2 minutes.

Both RaC and RaB accumulate in the kidneys. Liver, lungs, and femur contain slightly more than average on a per-gram basis, while spleen,

[3] These calculations were done by Jerome Cornfield.



TEXT-FIGURE 3.—RaA + RaB content of the blood.

muscle, skin, and intestine collect slightly less than the average amount, and testis, salivary gland, and fat have a low content, as seen in table 2. All tissues reach their maximum radioactivity in the first 5 minutes, except the kidney, which has its highest content at 15 minutes. As in the blood data, the scatter in the tissue data is great, especially for the liver.

RaB and RaC disintegrate rapidly and, therefore, table 2 does not present a true picture of the distribution of the nonradioactive isotopes, bismuth and lead, during the first 2 hours after intravenous injection. A correction for decay taken from the tables of Meyer and Schweidler (*10*) has been made and applied to the amount of radioactivity in the whole tissue divided by the amount injected into the animal (table 3).[4] As the intestines were weighed with fecal matter, a correction for the content was made. It is evident that the kidney is still accumulating the two isotopes of bismuth and lead at 120 minutes after administration, although more slowly than at first. The content of the liver, lungs, and femur remains quite constant during this 2-hour period, while the muscle and skin lose radioactive products after the first 5 minutes. Moreover, the appearance in the blood of additional material at 2 hours, as shown in table 3, is evident and is explained by the fact that the kidneys are not accumulating the products given up by the skin and muscles as rapidly as in the beginning. The total amount of radioactivity is well accounted for in all but the case of RaC at 30 minutes.

[4] The relative weights of various tissues were taken as: Skin, 16.5 percent; muscle, 50.0 percent; skeleton, 6.3 percent; brain, 0.9 percent; liver, 5.3 percent; lung, 0.72 percent; kidney, 1.35 percent; spleen, 0.9 percent; blood, 7.6 percent; intestine, 5.25 percent; salivary gland, 0.61 percent; abdominal fat, 2.4 percent; remainder, 2.2 percent.

TABLE 2.—*Radioactive content of various tissues following intravenous injection of RaA, RaB, RaC, and RaC'*

(a) $\frac{\text{C (t)/gm. tissue}}{\text{C (o)/gm. mouse}}$

Tissue	2′	5′	15′	30′	60′	90′	120′
Lung	[1] 1. 73 ±0. 09	2. 05 ±0. 02	1. 58 ±0. 14	1. 40 ±0. 11	0. 32 ±0. 01		0. 20 ±0. 06
Spleen	1. 17 ±0. 14	0. 74 ±0. 05	0. 64 ±0. 06	0. 52 ±0. 03	0. 45 ±0. 03		
Skin	0. 69 ±0. 04	0. 80 ±0. 05	0. 54 ±0. 04	0. 38 ±0. 01	0. 21 ±0. 01		0. 038±0. 003
Femur	1. 41 ±0. 11	1. 08 ±0. 18	1. 24 ±0. 05	1. 37 ±0. 11	0. 486±0. 05	0. 227±0. 04	0. 207±0. 05
Liver	1. 53 ±0. 20	1. 27 ±0. 16	1. 57 ±0. 11	1. 07 ±0. 08	0. 75 ±0. 09	0. 44 ±0. 16	0. 25 ±0. 03
Kidney	3. 02 ±1. 35	7. 88 ±1. 34	6. 9 ±1. 3	11. 53 ±0. 91	9. 42 ±0. 77	5. 76 ±0. 33	3. 11 ±0. 25
Muscle	0. 44 ±0. 05	0. 72 ±0. 04		0. 29 ±0. 01	0. 106±0. 012		
Intestine	0. 71 ±0. 10	0. 75 ±0. 09	0. 68 ±0. 01	0. 75 ±0. 05	0. 77 ±0. 001		0. 166±0. 006
Fat	0. 26 ±0. 02	0. 18 ±0. 001	0. 111±0. 004	0. 089±0. 01			
Blood	4. 45 ±0. 90	3. 73 ±0. 43	2. 40 ±0. 27	1. 70 ±0. 17	1. 07 ±0. 10	0. 67 ±0. 10	0. 45 ±0. 13
Salivary	0. 668±0. 062	1. 27 ±0. 22	0. 362±0. 030	0. 78 ±0. 02			
Testis	0. 321±0. 053	0. 352±0. 145	0. 176±0. 007	0. 163±0. 015			

(b) $\frac{\text{A+B (t)/gm. tissue}}{\text{A+B (o)/gm. mouse}}$

Tissue	2′	5′	15′	30′	60′	90′	120′
Lung	1. 76 ±0. 11	1. 57 ±0. 09	1. 36 ±0. 25	1. 60 ±0. 05	0. 24 ±0. 06		0. 007±0. 003
Spleen	1. 30 ±0. 19	0. 67 ±0. 04	0. 55 ±0. 08	0. 34 ±0. 02	0. 26 ±0. 03		
Skin	1. 09 ±0. 21	0. 74 ±0. 03	0. 37 ±0. 02	0. 20 ±0. 01	0. 045±0. 004		0. 007±0. 010
Femur	1. 76 ±0. 13	1. 41 ±0. 16	1. 23 ±0. 11	1. 24 ±0. 09	0. 42 ±0. 046	0. 200±0. 039	0. 27 ±0. 007
Liver	1. 68 ±0. 03	2. 12 ±0. 25	2. 51 ±0. 32	1. 42 ±0. 05	0. 58 ±0. 09	0. 27 ±0. 09	0. 17 ±0. 03
Kidney	3. 4 ±0. 6	5. 55 ±0. 54	8. 4 ±0. 8	2. 7 ±0. 3	4. 71 ±0. 14	2. 53 ±0. 15	1. 81 ±0. 41
Muscle	0. 353±0. 02	0. 39 ±0. 01		0. 235±0. 01	0. 037±0. 007		
Intestine	0. 860±0. 121	0. 572±0. 019	0. 502±0. 03	0. 477±0. 015	0. 278±0. 005		0. 057±0. 014
Fat	0. 254±0. 01	0. 260±0. 090	0. 119±0. 007	0. 045±0. 030			
Blood	3. 57 ±0. 042	3. 15 ±0. 33	2. 25 ±0. 25	1. 60 ±0. 12	0. 63 ±0. 10	0. 35 ±0. 07	0. 18 ±0. 07
Salivary	0. 640±0. 06	1. 05 ±0. 09	0. 355±0. 02	0. 143±0. 01			
Testis	0. 186±0. 046	0. 286±0. 072	0. 060±0. 025	0. 182±0. 027			

[1] Standard error.

TABLE 3.—*Radioactive content of whole tissues corrected for radioactive decay*

(a) $\frac{\text{C tissue}}{\text{C injected}} \times (F)$[1]

Organ	2′	5′	15′	30′	60′	120′
Kidney	0. 041	0. 106	0. 237	0. 191	0. 253	0. 300
Liver	0. 081	0. 067	0. 083	0. 069	0. 079	0. 096
Lung	0. 013	0. 015	0. 012	0. 012	0. 005	0. 010
Bone	0. 089	0. 068	0. 081	0. 106	0. 061	0. 093
Spleen	0. 010	0. 007	0. 006	0. 006	0. 008	-------
Muscle	0. 220	0. 360	[2] 0. 259	0. 178	0. 105	0. 069
Skin	0. 114	0. 147	0. 092	0. 077	0. 069	0. 043
Salivary gland	0. 004	0. 008	0. 002	0. 002	-------	-------
Blood	0. 338	0. 280	0. 189	0. 158	0. 185	0. 243
Intestine	0. 067	0. 070	0. 066	0. 070	0. 142	0. 144
Testis	0. 002	0. 003	0. 001	0. 001	-------	-------
Fat	0. 006	0. 004	0. 003	0. 003	-------	-------
Total	0. 985	1. 135	1. 031	0. 873	0. 907	0. 968

(b) $\frac{\text{A+B/tissue}}{\text{A+B injected}} \times (F)$[1]

Organ	2′	5′	15′	30′	60′	120′
Kidney	0. 047	0. 085	0. 167	0. 166	0. 296	0. 542
Liver	0. 091	0. 126	0. 196	0. 162	0. 143	0. 200
Lung	0. 013	0. 013	0. 016	0. 025	0. 008	0
Bone	0. 114	0. 100	0. 140	0. 168	0. 122	0. 178
Spleen	0. 012	0. 007	0. 010	0. 007	0. 011	-------
Muscle	0. 180	0. 220	[2] 0. 251	0. 252	0. 086	-------
Skin	0. 183	0. 138	0. 091	0. 071	0. 034	0. 024
Salivary gland	0. 004	0. 007	0. 003	0. 002	-------	-------
Blood	0. 278	0. 269	0. 253	0. 263	0. 216	0. 304
Intestine	0. 083	0. 060	0. 069	0. 096	0. 121	0. 118
Testis	0. 001	0. 002	0. 007	0. 003	-------	-------
Fat	0. 006	0. 007	0. 001	0. 002	-------	-------
Total	1. 012	1. 034	1. 204	1. 217	1. 037	1. 366

[1] Correction factor for decay.
[2] Interpolated.

With such uneven distribution in the tissue of the short-lived decay products, a great difference in dose to various organs occurs as seen in table 4. Here the energy absorption has been calculated for a dose of 0.016 mc./gm. of the radon solution. The total absorbed energy has been calculated (*8*) to be 4.17×10^4 ergs per gm., or 1.04×10^6 ergs for a 25-gm. mouse; this corresponds to 450 r X-radiation. The tissue doses are compared to this average dose in the fifth column.

Finally, the total energy absorbed in each tissue, as determined for a 25-gm. animal, is noted in the last column of table 4. The total absorbed energy has been surprisingly well accounted for. The dose administered to the intestine has been corrected for the weight of the contents, which averages about 44 percent of total weight (table 4).

TABLE 4.—*Radiation dose from RaA, RaB, RaC, and RaC′*

Tissue	ergs/gm.			ergs/gm.	ergs/gm.	25-gm. animal ergs per total tissue
	A	B	C+C′	Total	Average/ergs/gm.	
Lung	498	838	55, 860	57, 196	1. 37	10, 295
Spleen	86	850	54, 140	55, 076	1. 32	12, 392
Skin	150	229	18, 180	18, 559	0. 45	76, 649
Femur	129	1, 020	57, 856	59, 005	1. 42	92, 639
Liver	202	1, 440	51, 622	53, 264	1. 27	70, 841
Kidney	429	21, 900	561, 700	584, 029	14. 03	196, 818
Muscle	27	271	6, 227	6, 525	0. 16	81, 563
Intestine	99	720	183, 500	184, 319	4. 41	241, 563
Fat (abdominal)	24	140	4, 627	4, 791	0. 11	2, 875
Blood	2, 470	1, 470	109, 363	113, 303	2. 72	215, 276
Salivary gland	334	171	16, 000	16, 505	0. 40	2, 508
Testis	6	87	16, 920	17, 013	0. 41	3, 062
Total						1, 006, 481

DISCUSSION

Whenever radioactive samples are measured, the question of counting error arises. For the most part, the tissues were quite active and the counting rate varied roughly from 10 to 40 counts per second with background of about 1 per second. All tissues except the kidneys were low at 2 hours, and the testes and salivary glands were low at all times. The statistical error in counting for a single observation varied, then, from 7 percent to 3.5 percent; but as each tissue was observed for 20 minutes, the statistical error becomes, respectively, about 0.9 percent to 0.45 percent. The scatter in the data is not explained by the statistical error in counting.

The tissues are not all the same volume, nor are they the volume of the standard. Thus, geometry introduces a further error that was not estimated but which would be the same for all observations on a particular tissue. The large scatter can then be assumed to be a biologic phenomenon that is inherent in the mechanism of uptake and elimination of heavy-metal ions.

The possibility exists that radiocolloids were formed [Hahn (*11*)]. Dobson *et al.* (*12*) found that rare-earth colloids (especially zirconium) of large particle size have a biologic half life in the blood of 30 seconds to 1 minute, and those of a smaller particle size had a half life in the blood of 30 to 80 minutes. Since the first component in the elimination of RaC from the blood gave a half life of about 8 minutes, and since a still faster component would not have been absorbed, a RaC colloid might be considered to be the size of Dobson's large particles. The small amount of RaB and RaC present, however, is not conducive to large particle formation. Furthermore, the distribution of these rare-earth colloids did not depend upon chemical composition but on size alone. The large rare-earth colloids accumulated in the liver and spleen; the smaller in bone marrow and spleen. Both the RaB and RaC concentrated in the kidney.

The extracellular ions such as sodium, chlorine, bromine, and potassium (*13*), which do not form radiocolloids, diffuse from the blood rapidly and

in 40 minutes reach an equilibrium condition in which they are distributed homogeneously throughout the extracellular fluid. This behavior is also very different from that of the heavy metals. Since iodo-albumin (*14*), a colloidal protein, remains in the blood, indications are that RaB and RaC do not form colloids with the blood proteins.

Both bismuth and lead are well known to concentrate in the kidney in the acute phase. This may be a chemically preferential concentration. Plutonium (*15*), polonium (*16*), and radium (*17*) do not concentrate preferentially in the kidneys, however, but in the bone or spleen (polonium). Studies using these heavy metals were not started for ½ to 1 day after their administration, and comparatively large amounts conducive to colloid formation were given.

The total amount of A, B, and C in the mouse is well accounted for at most times. This is surprising in view of the variation in data. The high values for the total A + B may indicate an improper fit of the blood curve. Although the total C is low at 30′ the higher value at 120′ shows that any large amount of material has not been excreted.

The sum of the dose calculation is 3 percent below the total expected dose. This is a surprisingly good check. While the discrepancy may be accounted for in the elimination of RaC at 2 hours, the evidence of sufficient elimination is not strong enough to take the lower value of the dose in the comparison study. Most tissues get about the average amount of radiation but skin, muscle, and fat get less, and the kidney gets 14 times the average.

In view of these findings, the outcome of the comparison of the LD–50 is difficult to understand, and the question of validity of such a comparison arises. The α particles are not distributed homogeneously, and further concentration of dose occurs from the shortness of the particle track which is 70 μ in tissue.

There is a slight difference in slope of the regression lines (text-fig. 1), that for the alpha particles being somewhat steeper than that for the X-radiation. This may be due to chance but could be accounted for by the finding that the kidneys concentrate 14 times more than the average tissue. Although histologic examination of the kidney during the acute injury phase does not show morphologic changes, changes in the function of the kidney that are not detectable by histologic examination, may well be responsible for the greater slope of the regression line for the alpha radiation. In other words, the cause of death might be different in the two cases.

Nevertheless, the LD–50 as found in our experiments seems to depend upon the total number of ionizations, regardless of distribution, and not on specific ionization. The discrepancy between the finding and those on small biologic entities in which specific ionization plays an important role may perhaps be accounted for in the following way: In small biologic entities (such as bacteria, spores, seeds, and cells in cell culture), the primary effects of irradiation seem to be responsible for irradiation injury or death. In whole animals like the mouse, it is not the primary effects,

which disappear rapidly, that cause the ultimate establishment of the injury but the systemic secondary reactions provoked by the sum of the primary effects.

Similar conclusions could be drawn from the comparison of the effect of whole-body X-irradiation on tumor regression with that of α particles (*18*). Here equivalent energy doses produce the same degree of tumor regression. Although the tumor growing subcutaneously received far less than the average dose (½ to ⅓), the discovery of the synergistic reaction of whole-body radiation with local irradiation or tumor regression excludes one from concluding at this time that the α particles were actually more effective than X-radiation in tumor destruction. In addition, it could be shown (*18*) that for equivalent energy doses the effect on the picture of the circulating blood was quite similar for the two radiations.

Gray, on the other hand, found the efficiency of the neutron over X-ray increased to be 24 in the in-vitro studies on a rat lymphosarcoma (*8*) whereas the neutron efficiency had been 9 times that of the X-radiation in-vivo studies. He attributed this to a systemic effect. However, the whole animal was not irradiated here, but received scattered irradiation.

Finally, our data are in line with the findings of Zirkle on the relative biologic effectiveness of neutrons in dependence upon their linear energy transfer (linear ion density). He pointed out to us[5] that the relative biologic effectiveness as studied for the LD–50 increases with linear energy transfer and reaches a maximum for fast cyclotron neutrons (linear energy transfer 12Kw/μ). For fission neutrons (linear energy transfer 43Kw/μ) it decreases again. Accordingly the relative biologic effectiveness might continue to decrease for still greater linear energy transfer. Our data on the LD–50 for α radiation (linear energy transfer 110 Kw/μ) indicate such a decrease.

SUMMARY

The 30-day LD–50 X-radiation was compared with that of the α particles emanating from short-lived decay products of radon injected intravenously in strain A male mice. The integral dose of X-radiation necessary to produce 50 percent death was found to be 1.42 times that of α particles. Regression curves are given.

The distribution of short-lived decay products of radon was followed for the 2-hour period following injection. The distribution of the dose was calculated.

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A SPONGE MATRIX METHOD FOR TISSUE CULTURE. FORMATION OF ORGANIZED AGGREGATES OF CELLS *IN VITRO* [1] [2]

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INTRODUCTION

This is a report on the development of a short-term tissue culture method in which cells, and cell aggregates, growing into a sponge, may produce a basic tissue pattern having some similarity to the pattern observed *in vivo*. The living culture is studied by direct observation. After fixation and staining it is studied in detail using histologic techniques.

The established procedures of tissue culture by which cells grow on the surfaces of the media as sheets are of somewhat limited usefulness in the study of the histogenesis of normal and neoplastic tissue, and in the diagnosis of many tumors. While valuable information has been obtained in the study of tumors of the nervous system (*1*, *2*, *3*) and of some tumors of mesenchymal origin (*4*, *5*, *6*), the application of tissue culture to the diagnosis of carcinoma has usually been unsatisfactory. Cells of carcinoma, and epithelial cells in general, grow in tissue culture usually as sheets, in mosaic patterns, on the surfaces of the medium. Their appearance, when examined in the living culture, or after fixation and staining, is quite different from the patterns seen *in vivo*. The pathologist, however, in making a diagnosis, generally relies on the size and structure of neoplastic cellular aggregates and their relationship to the stroma, as well as on the morphology of the individual tumor cells. For this reason it is desirable to reproduce this structure and relationship if tissue culture is to be utilized successfully as a diagnostic aid.

GENERAL METHOD AND MATERIALS

Commercial cellulose sponge [5] was cut into pieces measuring approximately 8.0 by 5.0 by 1.0 mm., and thoroughly washed by boiling three times, for a total of 90 minutes, in distilled water prepared in an all-glass still. The pieces were then immersed in acetone, ether, and absolute alcohol, successively, for 15 minutes in each. Triple boiling with glass-distilled water was then repeated.

[1] Received for publication August 29, 1951.

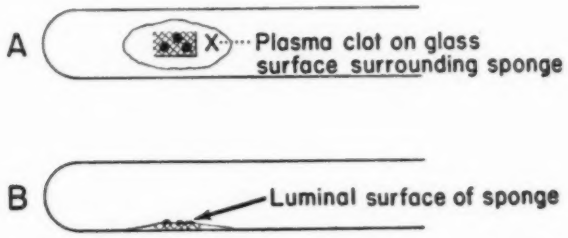
[2] Presented, August 22, 1951, at the Course in Tissue Culture given by the Tissue Culture Association in Cooperstown, New York.

[3] With the technical assistance of Thomas Stark.

[4] National Institutes of Health, Public Health Service, Federal Security Agency.

[5] "Plex," made of Dupont Cellulose Sponge by the American Sponge and Chamois Company, Inc., 51 Ann St. New York N. Y.

A piece of sponge was placed in a 1.5 by 15.0-cm. pyrex culture tube, about 2.0 cm. from the bottom. One to four fragments of tissue, each measuring between 1.0 by 1.0 by 1.0 mm. and 1.5 by 1.5 by 2.0 mm. were placed on the exposed surface of the sponge. A single drop of chicken plasma and one of diluted chick-embryo extract (20–25 percent) were placed on the tissue fragment and sponge. After clotting, when the fragments were firmly adherent to the sponge, 6 drops of liquid nutrient mixture were added (text-fig. 1). The tubes were then incubated at



TEXT-FIGURE 1.—A: Culture tube showing the luminal surface of the sponge from above, through the opposite side of the tube. B: Culture tube showing the lateral surface of the sponge.

37.5° C. in a roller drum at 15 revolutions per hour. Subsequently, three times weekly until the culture was interrupted, the liquid nutrient was removed and replaced by fresh fluid.

Two liquid nutrients were used in this study. One consisted of one part of 50 percent chick-embryo extract, two parts of horse serum,[6] and two parts of balanced salt solution (Earle's).[7] The other consisted of the same components, in the order given, in the proportion of 1:2:4. Each liquid nutrient supported the growth well.

The progress of growth in the sponge was followed by examining the culture directly at × 60 and × 100. At first the interstices of the sponge were optically clear, and the sponge elements had precise margins. By the end of 7 days, the explants were usually flattened and appreciably smaller. The interstices of the sponge, on the side in contact with the explant, became filled with granular and opaque material. Spindle-shaped cells, and sheets and cords of epithelial cells, were identified in many areas. Plasma clot liquefaction was seen after 5 to 10 days in multiple small foci, around areas of dense cellular accumulations. Many of these lacunae were continuous with the luminal surface of the sponge.

At varying intervals, from 2 to 4 weeks, when many interstices were filled with cells so that they were almost opaque, the sponges were fixed, dehydrated, embedded in paraffin, sectioned serially at 6 microns, and stained. The fixatives used were Zenker-formalin and Zenker-acetic acid; the stain was hematoxylin and eosin. A few sponge cultures were pre-

[6] The horse serum was kindly furnished by Dr. Wilton R. Earle of the National Cancer Institute.

[7] Earle, W. R., *et al.* Production of malignancy *in vitro*. IV. The mouse fibroblast cultures and changes seen in the living cells. J. Nat. Cancer Inst. 4 : 165-212, 1943.

pared as total mounts, after fixation in formalin-Ringer solution (*7*) and staining with Earle's hematoxylin (*8*).

OBSERVATIONS

Mammary Adenocarcinoma of a Mouse

With Cellulose Sponge

A mammary adenocarcinoma of a mouse, C3HBA (*9*), was studied.[8] A series was set up in which three or four tissue fragments were placed in each of four cultures. After 13 days of incubation, they were fixed in Zenker-formalin solution, embedded in paraffin, sectioned, and stained.

The original tumor from the donor mouse (fig. 1) was composed *in vivo* of compact acinar structures of small epithelial cells. Very rarely, a group of larger paler cells was seen surrounding a small glandular lumen. The outgrowth in the sponge matrix (figs. 2, 3, and 4) showed multiple areas of organized aggregations of cells that bore a distinct resemblance to the basic pattern of the original tumor. These areas were usually associated with marked clot liquefaction. Many glandular structures with distinct lumina were seen, along with smaller acinous clusters of cells in which lumina were not identified. Stromal elements were associated with some of the glandular structures. These were usually sparse, and consisted of spindle-shaped fibroblasts and a smaller number of histiocytes.

The cellulose sponge matrix was easily recognized in the hematoxylin-and-eosin preparations. It was a distinctive light orange color with fine black granularity, readily differentiated from the pink color of cytoplasm and fibrin. In the black and white photomicrographs, although the sponge is difficult to recognize, it can be identified by its faint gray color with irregular darker stippling.

The tissue patterns of the mammary tumor in sections of the sponge varied from sheets of epithelial cells to more complex glandular structures. A series of probable intermediate stages could be recognized, the process apparently being associated with progressive plasma liquefaction. The sheets grew on the surface of the plasma clot, either in contact with sponge or adjacent to a zone of liquefaction. As the liquefaction advanced, the sheet became retracted and angulated as it draped over itself and the supporting sponge. Acinar elements appeared in the zones where cells were concentrated (figs. 5, 6, and 7).

Without Sponge

The tumor was also studied in plasma clot without sponge. This could be done in an explant that was oriented on the sponge so that part of it extended onto the thin plasma clot lining the adjacent glass surface. Good growth consisting of barely visible sheets of epithelial cells could be

[8] Tumor-bearing mice were supplied by Dr. Ivor Cornman, The George Washington University Medical School, who obtained the tumor from Dr. M. K. Barrett, National Cancer Institute, in December 1950, in its 88th generation in C3H mice. The tumor, described below growing in sponge matrix, was inoculated in a strain C3H mouse, intramuscularly in the lumbar region, 22 days before it was removed for tissue culture, and was in the 98th transplant generation.

found in these areas. No tissue patterns were seen on the glass surface that were clearly comparable to those obtained in the sponge section.

Growth in plasma clot cultures 1.0 to 1.5 mm. thick was observed with fragments of mammary adenocarcinoma C3HBA from another mouse. In a series of six tubes, each with three or four tissue fragments, excellent outgrowth of epithelial cells was noted. The pattern of growth in the first week of cultivation was that of a thin sheet of epithelium spreading out on the plasma surface. In some areas spindle-cell proliferation into the plasma clot was noted. Liquefaction of the clot had begun by the seventh day of growth, and was followed by distortion of the epithelial sheets. The formation of spherical and oval masses of cells was observed in parts of the retracting sheets in the liquefied areas. Some masses were attached to the glass wall of the tube, others to the margin of the intact plasma clot. It was impossible to identify organized tissue in these cell groups. However, the presence of acinar structures was suggested by smaller round areas within these aggregates, some of which were of increased and others of decreased optical density.

A few plasma clot cultures without sponge were fixed and stained lightly with Earle's hematoxylin. The cell aggregates were completely opaque, although individual cells on the glass surface stained faintly.

Liver From the Chick Embryo

With Cellulose Sponge

In a series of cultures, two to four fragments of liver from a 9-day-old embryo were placed in five tubes. Direct observation showed good growth, particularly of closely packed spindle-cell elements, within 5 to 7 days. One culture was interrupted on the 9th day after incubation, two on the 12th day, and one each on the 14th and 21st days. Total mounts were prepared of the 9- and 14-day-old cultures. These were fixed in formalin-Ringer solution and stained with Earle's hematoxylin for 15 minutes. Both cultures stained intensely black, but after partial decolorization in distilled water acidified with hydrochloric acid, the surface of the 9-day-old culture could be examined satisfactorily using intense transmitted light. It consisted of a dense meshwork of plump spindle-shaped and stellate cells. The 14-day-old culture remained opaque after decolorization.

The other three cultures were serially sectioned. In the 12-day-old cultures, spindle-shaped cells made up an estimated two-thirds of the proliferating mass; the remaining third consisting of epithelial cells in varied patterns. Mitoses were seen in both spindle-shaped and epithelial cells. In some areas, usually at the periphery of the sponge, proliferating epithelial cells formed definite cords, irregularly oval masses, and glandular structures. In many large areas, especially in the central part of the sponge, the interstices were filled with masses of necrotic epithelial cells. In the 21-day-old culture, 4 days before interruption the side of the sponge previously bathed by the liquid nutrient became oriented against the glass surface. Except for a few areas of cell proliferation and gland

formation about the margin of the sponge, all the epithelial cells were necrotic. The viable stromal cells were irregularly oval or rounded, rather than spindle shaped.

Without Sponge

The pattern of growth of the embryonic liver in thin plasma clot was observed directly, as cellular proliferation occurred from the sponge to the adjacent clot covering the glass surface. On the surface of the plasma the growth was composed of a thin sheet of epithelial cells having a fine granular cytoplasm. Within the substance of the clot there were anastomosing, plump spindle cells with large oval nuclei.

Mixed Tissues From the Chick Embryo in Sponge Matrix Culture

A series of 10 cultures was prepared using tissues from a 10-day-old chick embryo, with 3 or 4 tissue fragments on each sponge. Various tissues including liver, heart, chorioallantoic membrane, sclera, retina, and lumbodorsal muscle were used, and mixed at random. Growth was vigorous in each sponge, and by the end of 10 days the inocula disappeared completely from the sponge surfaces. Single sponges were fixed on the 5th and 9th days of growth, the remaining 8 on the 19th day. All were serially sectioned. The 5- and 9-day-old cultures showed growth of epithelial and mesenchymal cells into the sponge spaces, but tissue formation was not extensive.

Each of the 19-day-old cultures showed abundant cellular proliferation and areas of distinctive tissue patterns. The sponge interstices were almost entirely filled with tissue. In figure 8 the tissue pattern produced by liver epithelium is illustrated. The sponge elements here clearly provide physical support for these organized aggregations of cells. The epithelial cells show many glandular formations in their clusters. A supporting stroma can be recognized, as seen in figure 8, which joins the masses of liver cells to one another and to the cellulose sponge. In addition, the masses of cells are covered with a definite layer of endothelial-like cells.

The pigmented layers of the embryonic eye grew very well *in vitro*, with densely and lightly pigmented epithelial cells making up large areas of tissue formation in the sponges. In figure 9 is an area of intensely pigmented epithelium forming a pattern of interlacing cords. Figure 10 shows a focus of striated muscle that contains open channels lined with endothelium, between groups of muscle cells. In figure 11 is an area of angiomatous tissue with many endothelial-lined cavernous spaces. This tissue is probably derived from embryonic heart.

Other Types of Sponge

Two other types of sponge were studied as reinforcement for the plasma clot. They were natural "spongin" sponge, and gelatin sponge.[9] These supported growth, but had several deficiencies. Spongin sponge was

[9] Gelform, The Upjohn Company, Kalamazoo, Mich.

slowly digested by the mammary tumor studied. It was extremely brittle after infiltration with paraffin, and histologic sections of it were prepared with difficulty. Gelatin sponge, although easily sectioned, was digested by the tumor and by several of the embryonic tissues of the chick.

A few experiments conducted with cellulose sponge washed in water alone appeared to have, on direct observation, a slightly reduced initial growth rate. However, the growth of embryonic tissue, on histologic examination, after 19 days, was equal to that in sponges washed in fat solvents.

DISCUSSION

There are a few intrinsic limitations to the sponge matrix method as compared with the techniques for tissue culture in general use. It is impossible to follow the progress of individual cells in the living culture. Also, since it is necessary to kill the culture to study its structure, any program for viewing the changes in a tissue at different time intervals would require a large series of cultures.

At present no data are available regarding the ease or difficulty with which subcultures can be prepared from growth in sponge matrix.

The formation of tissue in plasma-clot culture has been described by several observers. Cameron and Chambers (*10*) identified acinar structures and keratin pearls in cultures of human carcinoma. Parker (*11*) described tubular structures in the marginal area of a culture of mammary carcinoma in the mouse, cultivated in plasma clot, using a single flask for 6 months. In our plasma cultures of mammary adenocarcinoma in the mouse without sponge, we observed the formation of spherical, relatively opaque masses of cells; it was, however, impossible to make a positive identification of organized aggregations of cells on direct examination of these masses. It is technically very difficult to make histologic sections of thin plasma clots, particularly in the presence of broad zones of liquefaction. On the other hand, a plasma clot reinforced by a secondary, more substantial matrix, such as the sponge, can be easily handled with the usual care employed in manipulating small surgical biopsies. When clot liquefaction occurs, the retracting margins of plasma are supported by the sponge. Tissue that has formed in areas of plasma liquefaction, and that is subsequently bathed in liquid nutrient, retains its attachment to the sponge, being thus preserved for histologic examination.

The presence of sponge elements, intersecting the plasma clot in random fashion, provides a multiplicity of surfaces for physical support of the growth. Dense cell aggregates can be obtained in a comparatively short time, and on histologic examination, the same morphologic criteria that are used on material taken directly from the animal can be applied to many cultures. Both cell and tissue were identifiable in cultures of embryonic liver epithelium, striated muscle, and mammary adenocarcinoma.

Further study may show that a wide variety of embryonic, neoplastic, and regenerating tissues can grow in sponge matrix forming cellular

aggregates, with identifiable cells and distinctive tissue patterns. If this should be the case, in-vitro growth in sponge matrix might be employed to advantage in a variety of studies, such as those involving cell accumulation, differentiation, modulation, tissue formation, and the relationship of structure and function.

Once the pattern of tissue formation in the sponge has been clearly characterized for particular types of malignant human tumors, it is reasonable to expect that the application of this technique could provide valuable adjuncts to the methods and criteria now used in pathologic diagnosis, and in the evaluation of the effects of therapeutic agents.

SUMMARY

A simple technique for studying proliferating cells in sponge matrix combined with plasma clot is described. Organized aggregates of cells were formed in the outgrowths of explants from chick embryo and from mammary adenocarcinoma in the mouse. In the latter many glandular structures with distinct lumina were found, along with smaller acinous clusters of cells. Growth in sponge matrix is briefly compared with growth in plasma clot alone.

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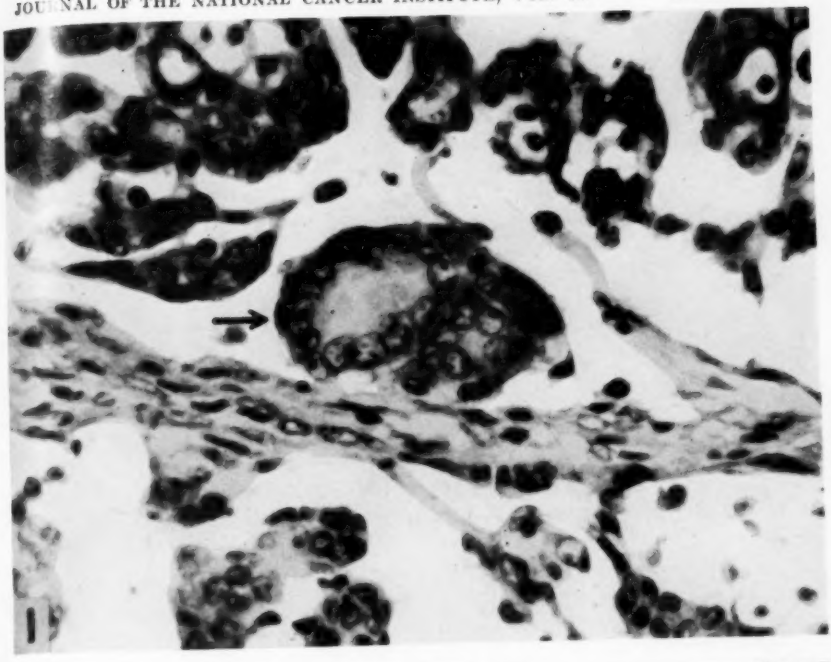
PLATE 57

FIGURE 1.—Donor mammary adenocarcinoma in a strain C3H mouse. Compact acinar structures and occasional larger glands (marked by arrow) are seen. Hematoxylin and eosin. × 340.

FIGURE 2.—Mouse mammary adenocarcinoma in cellulose sponge. Same tumor as in figure 1. The cellular aggregate, consisting largely of definite glandular structures, is covered by epithelium. Above and below the tissue the sponge (marked "S") appears faintly gray with fine irregular stippling. Hematoxylin and eosin. × 400.



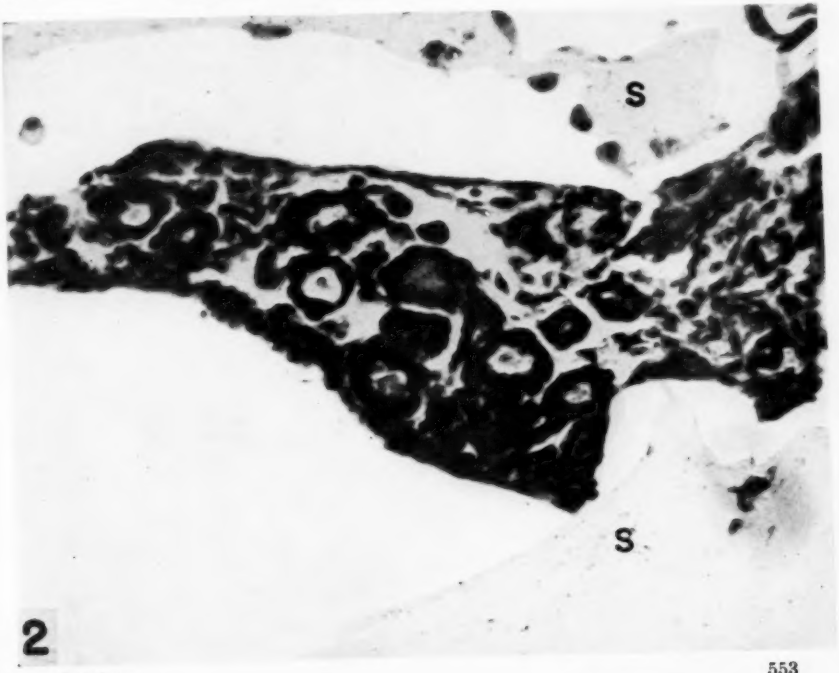


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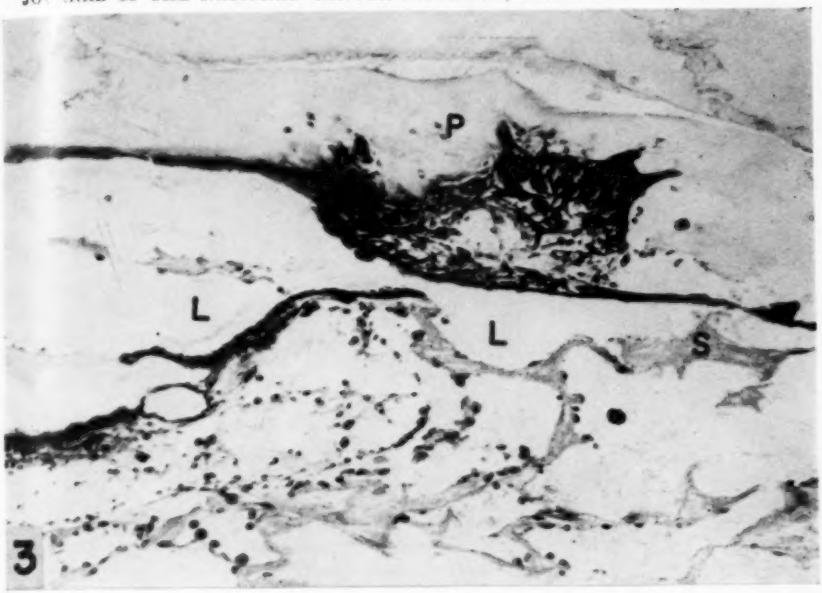
PLATE 58

FIGURE 3.—Mouse mammary adenocarcinoma in cellulose sponge (marked "S"). Section through a sheet of cells growing on the surface of the plasma, bordering a zone of liquefaction (marked "L"). The sheet is thickened in one area where organized glandular tissue has formed that extends more deeply into the intact plasma (marked "P"). Hematoxylin and eosin. × 75.

FIGURE 4.—Detail of glandular tissue formation seen in figure 3. One gland in the center of the field is composed of an inner layer of cuboidal cells and an outer layer of flattened cells (marked by arrow). Hematoxylin and eosin. × 300.


P
L
L
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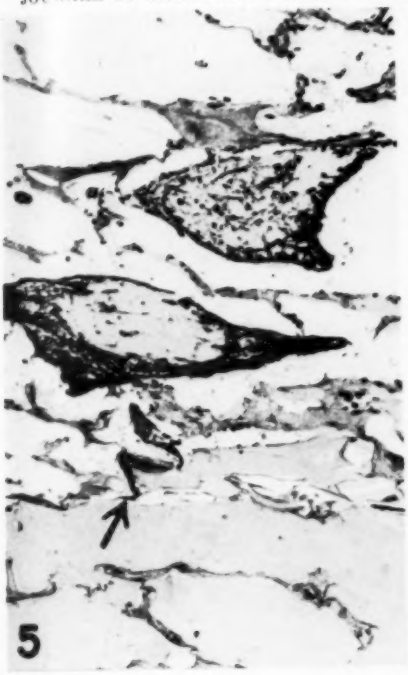
4

PLATE 59

FIGURES 5 and 6.—Mouse mammary adenocarcinoma in cellulose sponge. Section through sheets of epithelial cells growing on the surface of intact plasma. In several areas there is angulation of epithelial sheets (marked by arrows). In the spaces of the sponge where liquefaction is complete, more highly developed tissue masses have formed. Hematoxylin and eosin. × 100.

FIGURE 7.—Detail of a focus of angulation of an epithelial sheet. The tissue is covered by epithelium, and in its core are several glands. Hematoxylin and eosin. × 300.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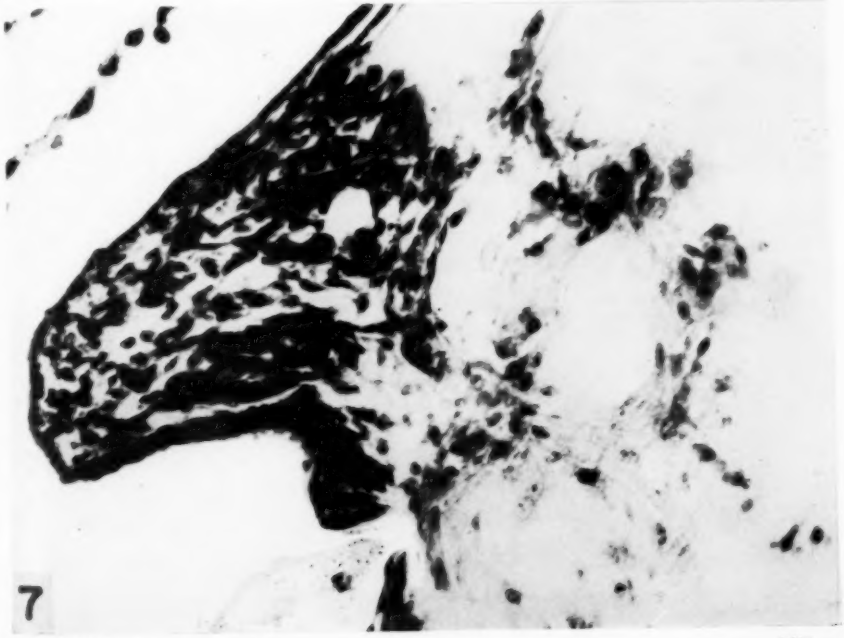
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PLATE 60

FIGURE 8.—Mixed chick-embryo tissues in cellulose sponge (marked "S"). Areas of tissue formation by liver cells contain many acinar groupings. Hematoxylin and eosin. × 400.

FIGURE 9.—Mixed chick-embryo tissues in sponge. Intersecting sheets of pigmented epithelial cells derived from embryo retina, appearing on section as "cords" of cells. Growth follows the contours of the sponge (marked "S"). Hematoxylin and eosin. × 400.

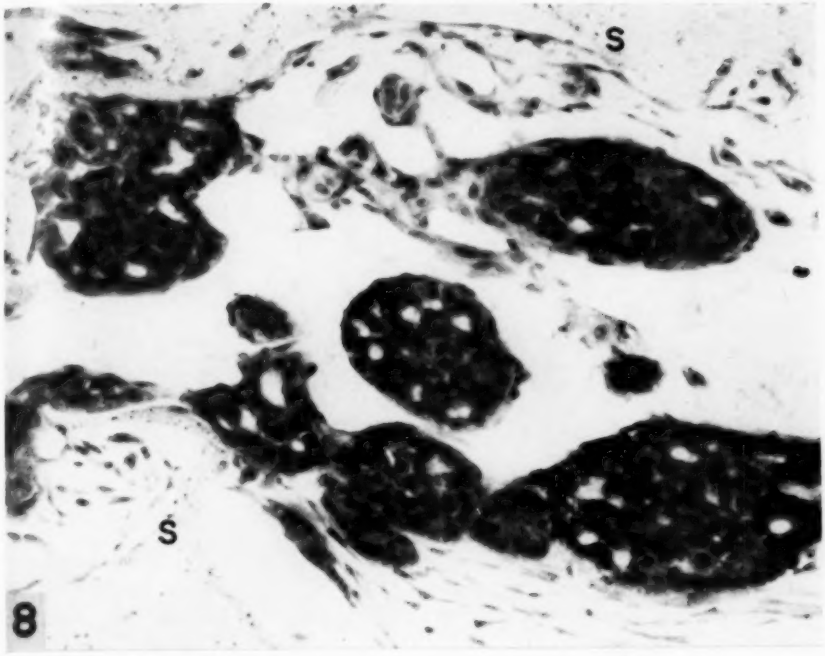
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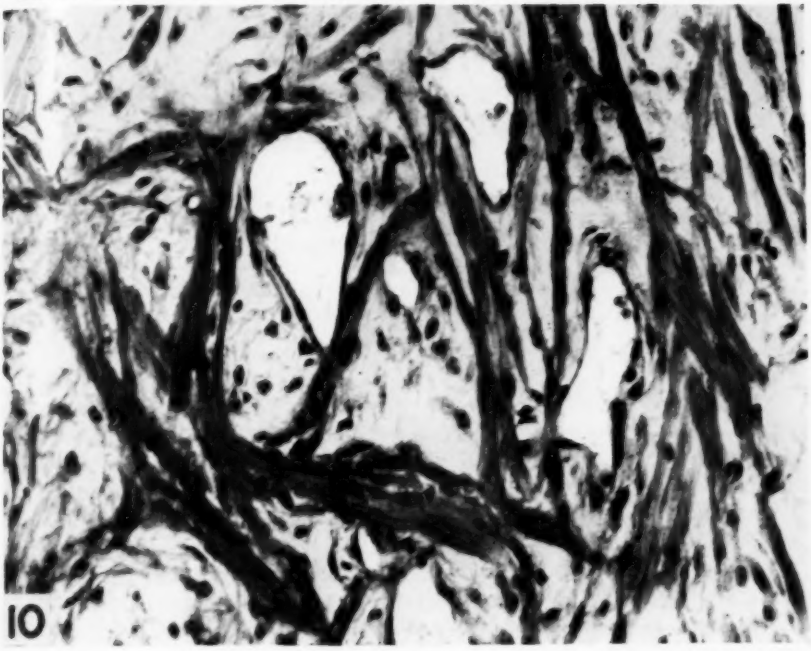
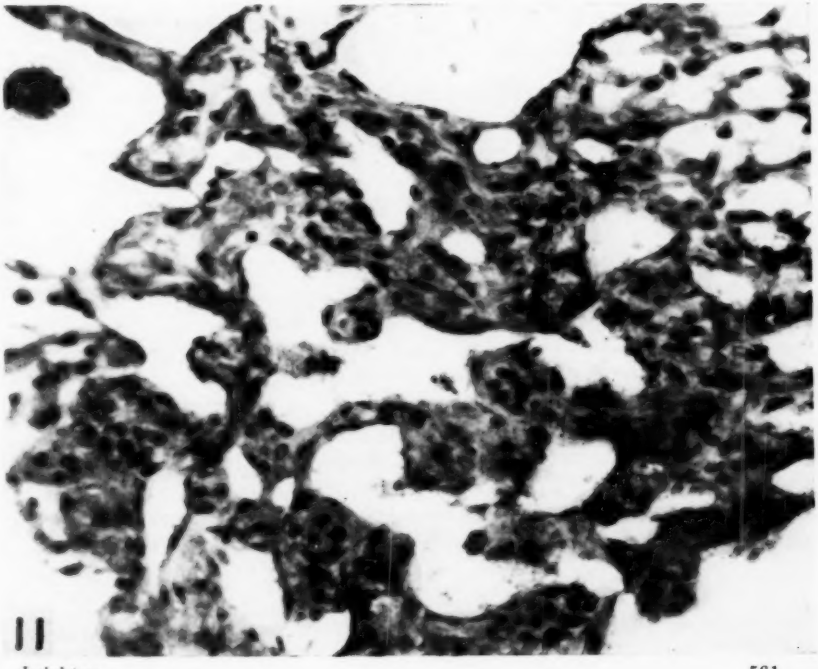
Plate

Figure 10.—Mixed chick-embryo tissues in sponge. Multinuclear striated muscle cells interspersed with endothelial-lined channels. Hematoxylin and eosin. × 400.

Figure 11.—Mixed chick embryo tissues in sponge showing anastomosing endothelial-lined channels probably derived from chick-embryo heart. Hematoxylin and eosin. × 400.





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INFLUENCE OF GLASS AND CELLOPHANE SUBSTRATES ON PROLIFERATION OF STRAIN L CELLS IN TISSUE CULTURE [1] [2]

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INTRODUCTION

In preparing replicate cultures by the procedure (*1*) described earlier, cellophane was uniformly used as the substrate. During these studies the observation (*2*) was made however, that after the single cell strain of strain L cells had been cultivated on cellophane (*3-5*) for a prolonged period, suspensions of the cells could be easily planted and would grow luxuriantly when subcultured to a glass substrate. Although these studies indicated that the luxuriance of growth of the cells cultured on glass appeared to be grossly comparable to that on cellophane, no quantitative comparison was made.

The purpose of the present study has been to determine, by enumeration of the cell nuclei, the relative luxuriance of growth of the strain L cells planted as a cell suspension on a pyrex-glass substrate and on perforated cellophane.

MATERIALS AND PROCEDURES

The materials and methods of the present study were those previously described (*1, 6*). T-12 culture flasks were used in all experiments.

A concentrated, coarse suspension of strain L cells was obtained from parent cultures in T-60 flasks (*7*). In some experiments the cells were suspended and stirred in the fluid culture medium (horse serum, chick-embryo extract and Earle's saline) or in undiluted horse serum; in others the suspension was prepared in Earle's saline to which 4 or 6 percent crystalline bovine plasma albumin had been added. The concentrated coarse suspension of cells was passed through a single or double-mesh platinum-alloy sieve and collected as a fine suspension in the stirring reservoir, where it was stirred for 10 to 15 minutes to obtain a final uniform cell suspension.

To obtain the desired dilution of cells measured volumes of the concentrated cell suspension were introduced into other stirring reservoirs containing known volumes of the suspending fluid. Aliquots of the cell suspensions were delivered in random order to glass or cellophane substrate culture flasks. The glass floor of the culture flask served as the glass culture substrate.

[1] Received for publication August 30, 1951.
[2] The authors are indebted to Mr. Marvin Schneiderman of the Biometrics Section of the National Cancer Institute for his evaluation of statistical data.

Cultures were carried for periods of 72 hours in some experiments and for 10 days in others. In the latter experiments the fluid culture medium was renewed every 48 or 72 hours as previously described (*1*).

When the cells were suspended in saline containing crystalline bovine plasma albumin, the cell suspension was added to the flask and then the horse serum and embryo extract were added in their correct amounts (2 vol. horse serum: 1 vol. embryo extract: 2 vol. saline) to produce a final total volume of nutrient culture fluid of 2.5 ml. Similarly, when undiluted horse serum was the suspending medium, Earle's saline and embryo extract were added in correct amounts to a total volume of 2.5 ml.

The cultures planted from each concentration of suspension constituted a group. Each group included a cellophane substrate set and a glass substrate set.

For the enumeration of the nuclei, random selection of cultures in the cellophane and glass sets was made from each group at the time of planting, at the end of 72 hours, and at the end of 10 days' growth. Average weighted differences in thousands of nuclei were calculated for 3 experiments run for 72 hours and 4 experiments run for 10 days. The *t* value was determined, and the probability that any difference in growth between glass and cellophane might be ascribed to chance was ascertained from the data collected and analyzed.

EXPERIMENTAL

Comparative Proliferation of Glass Surface and Cellophane Surface Substrate Cultures Grown 72 Hours

Procedure.—Three experiments with a total of six groups of cultures were set up and the cultures grown for 72 hours to test whether the final numbers of nuclei from initially replicate cultures of the pure strain of L cells varied when they were grown on a glass surface and on a cellophane surface substrate. The cells in the single group in the first experiment were stirred in horse serum. The suspending medium for the cells during stirring in the second experiment was the balanced saline with a 4 percent concentration of crystalline albumin. The suspending medium for the third experiment on three groups of cultures was the stock nutrient medium. The 80-mesh platinum sieve and the earlier design of stirrer which operated at 950 r. p. m. with the separate type burette unit were used in all three experiments.

Results.—Results of enumeration of nuclei on cultures grown on glass and cellophane for 72 hours are shown in table 1. The differences of the averages are weighted by a factor which takes into account the number of flasks used in computing the average. The averages, for cellophane and glass, experiment by experiment, do not differ significantly from each other. The probability that such a weighted difference as 36.25 or larger could have been due to chance alone, is between 40 and 50 percent. Therefore growth on glass for the 72-hour interval is not significantly different from the growth on cellophane. The differences that exist in this experiment, therefore, could be attributed to chance.

TABLE 1.—*Comparison of glass and cellophane as substrates. Results of 72-hour growth*

Experiment number	Glass			Cellophane			Superiority of glass over cellophane: weighted difference of the averages in thousands
	Number of flasks	Average number of nuclei per flask in thousands		Number of flasks	Average number of nuclei per flask in thousands		
		Inoculum	72-hour growth		Inoculum	72-hour growth	72-hour growth
1	3	901	2, 734	3	901	2, 679	+82. 5
2	3	270	793	3	270	731	+93. 0
3	1	983	2, 584	3	983	2, 725	−105. 8
	1	529	1, 598	3	529	1, 640	−31. 5
	1	306	1, 267	3	306	1, 071	+147. 0
	1	138	650	3	138	607	+32. 3

Average weighted difference in thousands of nuclei from 6 groups to show superiority of glass over cellophane as a substrate=+36.25.
t value=0.90.
Probability of a difference as large or larger due to chance is between 40 and 50 percent.

COMPARATIVE PROLIFERATION OF GLASS SURFACE AND CELLOPHANE SURFACE CULTURES GROWN 10 DAYS

Procedure.—Since no significant differences between cultures on glass and those grown on cellophane were observed in the short preliminary series grown for 72 hours, 4 experiments totaling 11 experimental groups (table 2) were made to determine if a 10-day growth interval did have a significant influence.

In the first experiment the cells were sieved through the 80-mesh platinum alloy sieve, and stirred with the 950 r. p. m. type stirrer in horse serum, chick-embryo extract and saline for a 2-hour interval.

In the second and third experiments the cells were sieved through the 80- to 150-mesh platinum-alloy sieve and stirred in balanced saline containing 6 percent crystalline bovine plasma albumin for a 1-hour interval.

TABLE 2.—*Comparison of glass and cellophane as substrates. Results of 10-day growth*

Experiment number	Glass			Cellophane			Superiority of glass over cellophane: weighted difference of the averages in thousands
	Number of flasks	Average number of nuclei per flask in thousands		Number of flasks 10-day	Average number of nuclei per flask in thousands		
		Inoculum	10-day growth		Inoculum	10-day growth	10-day growth
1	1	983	6, 836	3	983	5, 255	+1186. 0
	1	529	5, 366	3	529	5, 082	+213. 0
	1	138	3, 586	3	138	3, 718	−99. 0
2	3	--------	5, 989	3	--------	5, 351	+957. 0
	3	--------	5, 526	3	--------	4, 379	+1720. 5
	2	--------	4, 425	3	--------	2, 759	+1999. 2
3	3	1, 660	5, 163	3	1, 660	5, 290	−190. 5
	2	834	4, 845	3	834	5, 219	−448. 8
	2	172	3, 503	3	172	3, 073	+516. 0
4	3	150	4, 130	3	150	3, 568	+843. 0
	2	150	4, 765	2	150	3, 232	+1533. 0

Average weighted difference in thousands of nuclei from 11 groups to show superiority of glass over cellophane as a substrate=+640.42.
t value=3.01.
Probability of a difference as large or larger due to chance is between 1 and 2 percent.

The fourth experimental series varied from the second and third in that the cells were stirred in horse serum in the 450 r. p. m. design stirring unit.

Results.—Results of enumeration of nuclei in the glass and cellophane cultures at the end of 10 days are given in table 2. Weighted differences of the averages of the nuclei counts on 8 of the 11 groups indicate that growth on glass is superior to growth on cellophane. Of these 8 superior glass groups, 7 have higher weighted differences of the average than any of the 3 groups where cellophane was superior.

The probability of an average weighted difference of 640.42 or larger being accredited to chance alone is only 1 or 2 percent. The results indicate, therefore, that the growth on glass for the 10-day interval with this strain of cells is probably better than the growth on cellophane. This probability is extremely high.

DISCUSSION

The experimental data presented here are from a limited number of cultures of pure strain L cells allowed to proliferate for only an interval of 72 hours, and from a statistically more significant number of cultures grown for 10 days. The data on the cultures grown for 72 hours were collected early in the development of the equipment and methods used to prepare and handle replicate cultures. Since cultures on the glass substrate have to be handled with greater care than those on the cellophane, the effects of a gradual improvement in technical methods of handling would probably be more noticeable in maintaining continuous replicacy with cultures grown on glass than with those where many of the cells were under the protective cellophane film. The data from the later experiments seem to indicate that as techniques improved proliferation was better on glass than on cellophane.

For many studies in cell physiology a glass substrate for growth of cells has definite advantages over the use of a cellophane substrate. Preparation of the cellophane and the reorientation of the sheet frequently wrinkled or thrown down during centrifuging is eliminated. Since the complete role of the cellophane in the culture is not yet accurately defined, elimination of the cellophane in quantitative studies simplifies the experimental conditions and thus increases the accuracy of control of these conditions. The elimination of the cellophane from cultures under chemical or nutritional study excludes complications that arise from preferential absorption of certain constituents of the medium by the absorbent cellophane sheet.

From the data obtained in the present study with cell suspensions of strain L, the conclusion is reached that the cellophane substrate may be eliminated and quantitative procedures may be practically and accurately applied to produce cultures on the glass substrate at least as luxuriant as those grown on a cellophane substrate.

SUMMARY AND CONCLUSIONS

Strain L mouse cells of single-cell origin that had been grown on a cellophane substrate for an extended period and then planted as suspensions on substrates of pyrex glass and perforated cellophane have been quantitatively compared in their growth in T-12 flasks during 72 hours and 10 days.

Enumeration of nuclei in the cultures at 72 hours indicates no statistical difference in the growth of the cultures on these two substrates; in these experiments at 10 days there is a statistically significant advantage in favor of the use of glass as a substrate.

The quantitative procedures for preparing and handling replicate cultures and for enumerating their nuclei are easily and accurately applied to the glass substrate cultures.

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970188—52——15

ROLE OF THE CHEMOTHERAPY RESEARCH LABORATORY IN CLINICAL CANCER RESEARCH [1] [2]

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The study of the action of chemical agents in controlling malignant growths in the patient may be designated as research in applied science. How shall we regard the selection or synthesis of new agents, the investigation of their effects on tumors in experimental animals, and researches on the mode of their action? I propose that we regard this area of research as a *basic* science.

In addition to researches without any utilitarian objective, which are considered pure, academic, or fundamental science, there are many important research activities that we may call basic, for while they have a practical purpose, the objective is not an immediately useful one but rather the providing of a basis on which useful results may be constructed. After all, who cares whether mice are cured of malignant tumors? Dogs and horses are pets or are valuable in our economy, and there would be good reason for wanting to be able to treat tumors successfully in such animals. But not in the mouse!

Researches in this subject, from the point of view of their aim, lie between the applied research of clinical chemotherapy on the one hand and the academic or fundamental sciences on the other. In its operations, animal-tumor chemotherapy has many of the characteristics of the fundamental sciences but it has a clearly defined practical objective, namely, to provide knowledge on certain properties of tumors, to develop chemical agents that affect these properties, and to contribute in other ways so that clinical research in the chemotherapy of cancer may have a broad and sound *basis on which to build.*

It is not the function of the laboratory of chemotherapy to discover cures for cancer in patients. As I see it, this type of basic-science laboratory should be expected to advance knowledge in certain directions so that materials will be developed that will be worthy of study in patients.

[1] Received for publication September 12, 1951.

[2] Presented, on January 26, 1949, at the Sloan-Kettering Institute for Cancer Research, as one of the lectures in its *Seminars on Problems in Clinical Cancer Research.* The Sloan-Kettering Institute hopes to reproduce, by the photo-offset method, the manuscripts of all the lectures in that series and to distribute them, in booklet form, without charge. (Communication to the Journal of the National Cancer Institute from Dr. David A. Karnofsky, Chairman of the Seminar Committee.)

In preparing the lecture for publication in this Journal, some minor revisions were made in the text of the manuscript scheduled to appear in the projected booklet. Detailed data from the experimental work of the author and his associates, presented at the lecture on lantern slides bearing tables, charts, and photomicrographs, have been omitted. Some of the experimental data have since been published (see references); the rest of the material is being incorporated in manuscripts now in course of preparation for publication.

[3] National Institutes of Health, Public Health Service, Federal Security Agency.

Those chemical agents that produce results of sufficient interest in the cancer patient are then to be brought back to the laboratory for further study and modification in the light of the clinical effects observed. In many cases the toxicity will be found too high; the nature of the morphologic or functional damage that may be induced will need to be studied in the laboratory, and attempts made to prevent or minimize the undesired side reactions produced by each agent that is worthy of such effort. In other instances, the potency may be too low; here attempts may be made in the laboratory to enhance the potency, and to design for synthesis related compounds that might have higher activity. In other words, the work of the research laboratory in chemotherapy is not ended when it sends a sterile vial over to the clinician, with a label giving the formula and the concentration of the agent. This research laboratory is something more than a pharmacy shop. For, once a material is accepted for research in patients with advanced cancer, the second stage of the work of the research laboratory now begins, in intimate collaboration with the clinical investigator.

In discussing with Dr. Karnofsky, the Chairman of your Seminar Committee, the kind of material that should be presented today, I was relieved to learn that the general title of this series of seminars, *viz*, "Problems in Clinical Cancer Research," was not to be taken too literally. For, inasmuch as I am not a clinician, it would be rash for me to talk on clinical problems, particularly in such a famous clinical center as the Memorial Hospital. The purpose of these seminars, he pointed out, was to bring together certain lines of laboratory approaches and see what bearing they might have on clinical investigations. Accordingly, we agreed that it might be useful to assess the part that the laboratory can play in advancing chemotherapy research in cancer patients.

Problems in clinical investigation obviously are the responsibility of the clinicians, and it is to them that one ordinarily looks for guidance in such matters. Therefore, to put today's discussion in proper perspective, and to avoid even the appearance of presumptuousness, the title should be understood to mean "Problems in Clinical Cancer Research *as Seen from the Laboratory*." An alternative statement, which expresses even more precisely our subject for today, is "The Role of the Chemotherapy Research Laboratory in Clinical Cancer Research."

The care I have taken in defining the title and content is not just an exercise in literary hair-splitting. It is, rather, a necessary precaution if progress is to be expedited in a field so long regarded as one in which "fools rush in where angels fear to tread." Among the questions that might be considered with advantage, are:

First: from the point of view of clinical progress, is the chemotherapy laboratory likely to be an aid or a liability?

Second: if it is considered an aid, what should be the relationship between the scientists in the laboratory and the clinicians in the hospital?

Third: since it is blasphemous even to imagine a situation where a clinician is subordinate to a laboratory man, and if the relationship is to

be that of master and servant, what caliber of research scientist is likely to be attracted to, and continue to be satisfied with, the status of a servant?

Fourth: is it too visionary to suggest a picture in which the investigator in the clinic and the investigator in the laboratory regard each other with mutual respect as scientists, and collaborate on an equal basis?

To the egotistical and the cynical, these questions may appear simply as another example of a squabble for position and power. But there is far more to it than may appear at first sight, for accomplishment in research is not automatically assured by providing tall buildings, large budgets, fine blueprints of divisions and departments, nor even by big payrolls. Research is done by *people*. And if the intellectual and emotional environment is not wholesome, the more people there are in the organization, the higher the incidence of frictions. Many large industrial organizations are aware of such undesirable potentialities and have taken intelligent steps to foster good morale and a cooperative spirit.

In Bethesda, work has been begun on building a huge physical establishment. There will be expansion of the laboratories of the National Institutes of Health and establishment of 125 research beds for cancer patients. It is already apparent that much careful thought will need to be devoted to the relationships among the organizational units and among the human elements if such large ventures are to be productive enough to justify the large expenditures.

While much of the foregoing has applicability far beyond our particular aspect of cancer research, I have brought it into the picture because of its especial pertinence to chemotherapy. In any scientific field in which but little progress has been made there usually are, and there should be, widely differing ideas on how the outstanding problems should be tackled. In cancer chemotherapy, however, there has been, and there is even nowadays, the additional factor of highly charged emotional attitudes that hinder cool analysis of strategy and tactics.

From the historical point of view, research in chemotherapy of cancer, until quite recently, has been an unpopular field. The previous disappointments were so numerous, and they had extended over so many decades, that a feeling of defeatism was prevalent; there were few investigators, indeed, willing to waste their time and budgets on this subject. But the human need was great and pressing. So the field was pre-empted for many years by quacks and by well-meaning incompetents, to such an extent that an aura of disrepute came to hang over it.[4] It was consequently more difficult to secure budget for chemotherapy of cancer than for other aspects, or even for alleged aspects, of cancer research. Moreover, a serious investigator who devoted himself to work in this field ran the risk of having himself considered a bit disreputable or a bit "cracked."

Fortunately, however, there has been a striking change of opinion in the last few years. Optimism is vastly on the increase, and it is now respectable to engage in research on chemotherapy of cancer. In two of the

[4] See, for example, "Cancer and the Need for Facts. I. Frauds and Fables. II. Unestablished Remedies." Report of the Council on Pharmacy and Chemistry, J. A. M. A. 139: 93-98, 1949.

largest institutions, in the National Cancer Institute and in the Sloan-Kettering Institute, there are departments specifically devoted to chemotherapy. The Committee on Growth has added a Chemotherapy Section to its organization, with funds available for support of projects in this formerly neglected field.

This important change in attitude has come about because it has been demonstrated that there are at least a few chemical agents that are capable of affecting tumors, and because a small number of courageous and vigorous personalities persisted, until they succeeded, in getting wide recognition of the significance of the old leads and of the new ones. Encouraging as have been the results obtained here and in other institutions, they are not really satisfactory solutions. The most effective chemotherapeutic agents available today are either too toxic or they influence only a limited percentage of tumors even of restricted types, and the effects obtained, when they are obtained, are only partial or temporary. Nevertheless, they show that malignant tumors *can* be influenced with chemical agents, and they provide additional warrant for the search for more satisfactory ones.

It does not come within the purview of today's seminar to give a digest of the technical data on cancer chemotherapy contributed from various sources during recent years, particularly since this field has been the subject of two recent reviews (*1*, *2*). However, in discussing the relation of the research laboratory to clinical investigations in chemotherapy, I shall refer to work in my own laboratory in Bethesda for purposes of illustration.

In the Chemotherapy Section [5] at the National Cancer Institute a systematic program, on a limited scale, has been in progress. The scope of the program, the exposition of the several lines of work, and some of the progress reports, have been presented (*3–7*) from time to time by the various investigators in our group and in associated groups. We have on hand more than 2,000 chemicals that we have collected, prepared, or synthesized for screening against transplanted tumors in mice. About 1,000 of these have now been given a crude first screening. The selection of these agents was not a random one but was based on certain hypotheses, or hunches, if you will.

Of the first 1,000 agents about 950 yielded negative results. Some 50 [6] compounds, however, gave positive results. By positive I mean objective, reproducible tumor damage under the conditions of our screening. This, of course, does not mean that among these 50 compounds there is included "the cure for cancer"; all that it means to us is that, in the future, we would rather carry out further experiments with those of the compounds that gave positive results rather than with those that gave negative ones.

In the first stage of our screening, Sarcoma 37 is implanted into large numbers of mice of selected genetic types. When the tumor is 1 week old, a single subcutaneous injection of the compound under test is given,

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[5] Renamed, in 1951, the "Laboratory of Chemical Pharmacology."

[6] As of September 1951, some 3,000 chemical agents have been selected and screened, of which about 400 have yielded positive results.

contralaterally to the established tumor. The dose is selected so as to be just under the lethal. Our usual terminology is "the maximum tolerated dose" but, to avoid misunderstanding, I am stressing that we aim for a sublethal dose, *i. e.*, a dose of less than LD_{10}. The mice are all sacrificed within 48 hours after the single injection, and the tumors are examined grossly and microscopically for evidence of induced damage. Under these restricted circumstances, about 50 of our compounds have shown the capacity, in repeated experiments, of damaging Sarcoma 37.

During this past year we have been able to get under way further experiments with these positive compounds. In the subsequent stages of the program, the positive ones are investigated further. They are being examined for maximum tolerated dose levels in repeated injections, both in normal mice and in tumor-bearing animals. They are also tested against a variety of transplanted tumors such as carcinoma, lymphoma, leukemia, and melanoma. Toxicologic and histopathologic data are being collected with other species of animals, such as the rat, rabbit, and dog. The positive compounds, along with negative ones of closely related structures, are being examined for potency in damaging certain enzyme systems in the tumors and in normal tissues, and the chemical damage observed is being correlated with the histologic and cytologic evidence of induced damage, to obtain information on mechanism of action of the positive agents.

Other institutions, including yours, also have substantial programs of laboratory research in chemotherapy of cancer either in progress or in process of organization. The programs differ in the compounds under study, in the techniques used in evaluating the chemicals, and in other ways. But they all have in common the aim of contributing to the development of agents with possible clinical usefulness.

Now this effort and expense may all be very well, considered as a pastime to keep laboratory scientists from boredom, but may it not be wide of the mark? After all the labor is done, the compounds found to be positive in one laboratory or another may not be of any use in clinical practice. This brings us to the question: How can the research laboratory be of value for *clinical* researches in cancer chemotherapy?

Many answers have been given. A favorite one is: The chemotherapy laboratory can be of *no use* to the clinical problem, and it should not exist as a separate entity! However, there are increasing numbers of people who are convinced that the chemotherapy laboratory can help clinical investigation. There are some here this afternoon who, like myself, are of this opinion else they would be digging in another field. Let us see if we can crystallize the reasons for our opinion.

One way of focusing our thinking is to consider the objections so abundantly offered by those who view our operations with a jaundiced eye. These objections arise from three important theories, which might be designated as:

1) the "No Use Theory"
2) the "Lucky Accident Theory"
3) the "Great Genius Theory"

Let us consider the "No Use Theory." Interestingly enough, it is held by two widely divergent groups, for widely different reasons. One group consists of some "fundamental" or "pure" scientists; the other consists of some highly "practical" medical men. Time and again these "pure" science colleagues of ours have warned us that we are wasting our own time and our institutions' money by fumbling empirically in the dark, without rational bases for our chemotherapy experiments. They have advised us repeatedly to concentrate, with them, on elucidating the fundamental phenomena involved in normal growth and in neoplastic growth until, ultimately, there will have been revealed a thorough understanding of all the structures and functions involved. Such knowledge, they say, will enable us at some future date to devise intelligent, rational bases for experiments in chemotherapy. In the absence of this knowledge, and of reliably founded rationale, they say, our efforts are doomed to failure, and should be discontinued.

On the other hand some "practical" clinicians have said that "Man is not a mouse," that even should there be developed in some laboratory an effective chemical treatment for cancer in the mouse, there is no assurance that it would influence malignant tumors in the patient. An even worse danger, they have pointed out to us, is that some chemical which, unbeknownst to us, has great value for clinical cancer may be ineffectual against mouse tumors and may therefore be stigmatized by mouse experimentalists as negative and accordingly may be discarded, with tragic loss to humanity.

The sensible laboratory worker in cancer chemotherapy is fully aware of these *a priori* objections. He not only does not contest their cogency, but he plans his experimental attack in acute awareness of these considerations. In short, he takes them into account, in the terminology of the military strategist, as calculated risks. However, in contrast with the known and the probable risks, there are considerations that lead him away from the "No Use Theory."

In the first place, this school of "pure" scientists is actually unscientific in predicting that a problem cannot be successfully handled unless all the theoretical aspects are first worked out. The history of science abounds in instances of applied science, and even empiric technology, having yielded reasonably satisfactory answers to problems without prior mastery of the basic phenomena involved. Moreover, in addition to working out new solutions, applied science and technology have in many instances actually contributed in an important degree to fundamental science. Did knowledge of the role of para-aminobenzoic acid in bacterial metabolism lead to the synthesis and trial of the sulfonamides? Of course not! It was first found that the sulfonamides were remarkably effective. Then, when studies were carried out on the mechanism of their action, these empirically discovered therapeutic agents led to extension of fundamental knowledge of competitive chemical reactions in the metabolism of bacteria.

The case of penicillin is still more recent. It has a far greater range of potency than sulfonamides, and an almost negligible toxicity. Was penicillin developed because theoreticians had fully accounted for the mechanisms in drug-sensitive bacteria and spirochetes in such a way as to point to the advisability of trying this metabolite of this mold? To ask the question is to answer it. In fact, the empirical work in this field has resulted in great advances in knowledge of many topics in the fundamental sciences. And how about radium and X-rays? It is now about half a century since they have come into use. Do we, even now, know much about the mechanisms that determine radio-resistance and radiosensitivity? Did this type of knowledge precede or follow the employment of radiation in the treatment of cancer?

Numerous other instances might be cited, but we need not belabor the point. Our view must not be misconstrued as casting aspersions on the value of pure science or on the need for greater expansion of research in the pure sciences. There is really no conflict between pure science, on the one hand, and basic and applied science on the other. In fact it is not always easy, in operational terms, to distinguish one from the other. However one prefers to define them, and wherever one draws the borderline between them, it should be clear that traffic does not flow in only one direction, from fundamental to applied, as is so often held. There is actually a two-way stream, with the findings of basic and applied science constantly enriching fundamental knowledge.

Of course if more were known about the characteristics of malignant growths as compared with those of normal tissues, it would be possible to formulate working hypotheses and techniques for chemotherapy that would be more helpful, and we could expect progress to be much faster. Nevertheless, it is unsound to pontificate that new therapeutic agents for cancer cannot be developed until we learn the alpha and omega of growth processes.[7] Enough knowledge is already at hand to furnish leads for sound research in chemotherapy by a combination of empiricism and of theory.

As to the thunder on the right from some of the clinicians it is obvious that here, too, there is much cogency in their argument. The laboratory man, perhaps even more clearly than the clinician, realizes the hazards in transferring conclusions from one species of animal to another. The rat is a splendid animal for the study of vitamin D; the mouse is a poor one. The guinea pig is most helpful in studying the action of vitamin C, whereas the rat is congenitally uncooperative in this respect. Paint the skin of the mouse with benzpyrene and tumors are obtained in practically all of them in a short time. Yet this carcinogen has little potency for the skin of the rabbit. These animals are not only all mammals, they are all rodents. The competent laboratory investigator in cancer knows full well how risky

[7] In an entirely different field, that of the physiology of the "normal" individual, similar views have been expressed by Keys (8). In a paper presented shortly after this lecture was given, Keys stated:

"This is not to decry in the least the virtue of a more theoretical approach. Given enough knowledge of theory and quantitative information on the mechanisms, both of the problems discussed here could probably be more easily and surely solved. The point is that such knowledge is now lacking and, as a matter of fact, a good deal of empirical gathering of data, plus rigorous analysis, is necessary to create the wanted body of knowledge."

it is to transfer conclusions even from one rodent species to another, let alone to a primate.

Although the laboratory worker in chemotherapy fully agrees with the practical clinician on these hazards, he does not accept the "No Use Theory" because he is not satisfied with the alternative. The advice given by this type of objector is to confine screening in cancer chemotherapy to clinical material. Well, let us imagine that a clinical investigator can have at his disposal:

a) ten patients with the same type of tumor, biologically and microscopically, available for each experiment without the complications of other forms of treatment

b) a similar group of patients for each type of tumor and for each compound he wishes to study

c) appropriate numbers of similar patients as controls.

Overlooking the difficulties which would be encountered even thus far, where would such numbers of beds be provided for each of these clinical experiments in present-day overcrowded hospitals? And the expense? At a cost of at least $20 per day per research bed and accessory activities, it would amount to more than $5,000 per month for each compound.

Let us now stretch our imagination still further, and conceive a situation in which there are *no* bottlenecks, *viz:*

a) expenses are all provided

b) beds are always available

c) suitable patients with appropriate tumors of desired sites are available in any number needed

d) no objections are raised by the patients, their families, or their medical advisors

e) plenty of nurses are available

f) clinical investigators, residents, and interns are available and interested, in the necessary numbers and of the requisite caliber.

Let us thus suppose the millenium has arrived as far as the logistics for clinical investigation in this field are concerned. We now come to the $64 question: "*What chemical agents are to be screened* in these patients with incurable cancer?"

Many hundreds of thousands of chemical materials, both organic and inorganic, have already been described in the chemical literature. Some have been extracted from natural sources: inanimate, plant, and animal. Many more have been artificially prepared, either by conversion or by synthesis. Many millions of compounds more can be extracted from natural sources or synthesized. How is the clinical investigator to make a selection from all this chemical wealth and variety? Here is where the chemotherapy research laboratory can play an important role, *viz,* in the *process of selection* of agents for the clinical investigations.

Clinically, the most notable recent advance in cancer chemotherapy has been the work of Farber (*9-11*) and his associates at the Children's Medical Center in Boston on the effect of aminopterin and other compounds upon the course of acute leukemia in children. By now Farber's

findings have been repeatedly confirmed in various quarters. His achievement was an outgrowth of a preceding study, at that institution, in which folic acid preparations were administered to patients with various types of malignant disease including leukemia. In turn, *that study was based upon laboratory investigations in tumor-bearing animals, viz,* a) the well-known work of Lewisohn and his associates (*12*), extending over many years, in which mice bearing spontaneous mammary carcinoma were employed, and b) Farber's own experiences (*13*) on the behavior of transplanted sarcoma in folic acid-deficient rats. The point to bear in mind is that a large amount of work was first carried out with experimental tumors in laboratory animals, and that it was upon this basis that the clinical study was set up.

If experiments with animals and animal tumors are to be discarded, what is recommended instead? Systematic screening for cancer chemotherapy in patients is impractical. What is offered as the way out?

There is presented to us the second theory, the "Lucky Accident Theory." According to this line of reasoning, the problem is so complex and so difficult that it would take a tremendous effort in men and money and time to make significant headway in a frontal attack whereas, sooner or later, an agent or agents useful in clinical cancer will be discovered more or less by chance, as byproducts of other lines of investigation, or even by mere accident.

This may well be the case, but suppose the Lucky Accident doesn't happen for several more generations? What of the meanwhile? Moreover, suppose that lucky accident has already occurred! Occurred, that is, in the sense that a most valuable chemotherapeutic agent for cancer is already in existence and known chemically, but that its valuable properties for cancer are as yet unsuspected. Shall we wait for a Second Lucky Accident to reveal to us the biological properties of the chemical whose isolation or synthesis was a Lucky Accident in the first place?[8]

This brings us to the third theory, the "Great Genius Theory," which postulates that since so little is known in these matters, it takes the intuition of a genius to select the chemicals and demonstrate their potency. Lesser lights should not bother themselves or others. One trouble with this theory is the vagueness in defining the hallmark of a genius *on the make*. So often the recognition of genius comes long after success is achieved. How much support did the Curies get *before* they isolated radium? How readily did Pasteur get acceptance for the value of his epoch-making contributions to etiology and immunity in infectious diseases? And Ehrlich in his chemotherapy, how many years did it take for that battle to be won? In short, if geniuses were more plentiful, or if

[8] In a colorful talk entitled "Accident and Opportunism in Medical Research," Sir Henry Dale (*14*) stated:

"I hope, however, that my choice of a subject and citation of examples will not bring me under the suspicion of suggesting that accident is the principal factor of success in research of any kind, or of medical research in particular. Accidents of the kind which we have been discussing do not, in fact, happen to the merely fanciful speculator who waits on chance to provide him with inspiration. They come rather to him who, while continuously busy with the work of research, does not close his attention from matters outside this principal aim and immediate objective but keeps it alert to what unexpected observation may have to offer. I certainly do not believe that any research work of permanent value is done, or any discovery of importance soundly established, without a great deal of hard, systematic, and conscientious work."

somebody would tell us how to spot them *in advance* so we could invite them to solve this problem, we might perhaps relax and leave it to them. However, it is rather more likely that it will work in backward fashion, *i. e.*, if someone should win through to valuable achievement he will then have pinned on to him, *afterwards*, a lofty designation.

Let us suppose that there were combined in one encyclopedic individual all the knowledge that is required for successful achievement in this line of research: this hypothetical individual would, according to our present notions, have mastery of organic chemistry, cytology, pathology, biochemistry, pharmacology, *etc.*, as well as of clinical medicine. Even so, there would be severe limitations on his productivity for, single-handed, most of his tools would go unused most of the time. No musician, no matter how talented and versatile, can simultaneously play the violin, bassoon, and piano, or can substitute for an entire orchestra.

However, the advantages of having mastery of all these sciences reside in one individual may be approximated by a genuinely cooperative group. Such a group would consist not of one Master Mind dominating a staff of supertechnicians with doctors' degrees; it would be a real team of investigators who voluntarily associate themselves in such a working group because of the conviction that this is a more fruitful way of tackling the problem than by playing a lone hand. This does not, by any means, imply regimentation of their researches by some dictator; it is rather that the investigators, of their own volition and by common agreement, would choose the same direction for their various researches. There would be, in such a team, joint use of material and unpublished data, and free but friendly criticism of results and original ideas. These mature specialists would voluntarily dovetail their plans and their experimental work so that their findings, taken together, would have more meaning than had each man operated as a lone wolf.[9]

It is not easy to recruit such a group, nor is it a simple matter to arrange for its effective functioning in a spirit of harmony over long periods. Without harmony, the operations of such a team become disrupted. It is therefore essential constantly to bear in mind the personal reactions of the people, for the members of the team are human individuals and not pawns on the organization chart of a large institution. Each man must be considerate of his team-mates, and there must be quick and generous mutual appreciation and acknowledgment of the contributions of each man to the common goal.

Is it feasible to extend this pattern and these attitudes to relations among groups working in the same field but in different institutions? This is far more difficult to achieve than some imagine. To a considerable degree, informal cooperation has already developed among many groups in the field without any formalization in terms of official "coordination" or "pooling" from higher organizational echelons. But, curiously enough, it has bogged down precisely in those quarters that have been most vocal in advocating official coordination on a national basis.

[9] For a fuller presentation of the theme "Teamwork in Scientific Research," see the paper (*15*) with this title in the Official Publication of the Society for Personnel Administration.

The secret of success in promoting genuine cooperation among individuals and groups, in this country at any rate, appears to depend on several factors. In the first place it must be voluntary in nature. Secondly, if it is to flourish it must grow up from the grass roots and not be imposed from the "outside" or from "above." [10]

Thirdly, it must be envisaged not as collaboration between institutions as such but rather as between individual investigators who happen to be located in different institutions.

This last factor is a most important one. For, in fact, institutions or groups do not really work together; it is the *people* in one institution who cooperate with *people* in other institutions. All that administrations can do in this regard is to give such cooperation their blessing, and provide a favorable climate for it, but genuine and fruitful cooperation must emanate from the individual research men. And people do not collaborate successfully unless they have confidence in each other, confidence not only as regards technical competence but also as regards temperament and character.

The self-centered whose utterances give lip-service to the necessity for cooperation, but whose actions show that what they really want is the services of others without any corresponding help in return, defeat themselves and discredit the concept of genuine cooperation. Those who, in the name of quicker progress in an altruistic cause, demand that others turn into a common pool unpublished material and protocols but who, while making these demands of others, keep monopolistic control of their own chemicals and keep secret their own unpublished protocols, do not help develop a cordial spirit of cooperation among workers in the field; they only provoke resentment. Those institutions which lend themselves to the issuance of misleading publicity in newspapers and lay magazines giving the public the impression that they are responsible for work which was really done in other places, should not be astonished if the policy boomerangs by the building up of ill-will instead of good-will. In short, the old-fashioned virtues of intellectual honesty, consideration for others, and generosity to colleagues, even to those in other institutions are essential to the development and maintenance of a cooperative spirit. Incidentally,

[10] In one of the most peculiar policy statements in the history of cancer research in the United States, the "uncontrolled and illogical" enthusiasm with which investigators shift the emphasis of their researches was likened to the behavior of children at a zoo; it advocated coordinated *control* of funds for cancer research by persons who were not specified. One paragraph from this article (*16*) stated:

"Cancer research. It is now clear that the time is ripe for organized and cooperative efforts in the field of cancer research. The cancer problem as such cannot be directly compared with any industrial or commercial field, but there are certain principles which can be transferred from one situation to the other. Among these is recognition of the fact that well organized, long-time programs of research are more apt to lead to important results than are scattered, disoriented activities. One of the characteristics of cancer is that it requires uninterrupted study in order to observe its origin, development, and activity in relation to other normal functions of the body. While there have been, until the war, opportunities for exchange of views by research workers at annual meetings of the American Association for Cancer Research there has been little if any effort to develop continuing drives along broad lines that give promise of progress. *When some new and sensational discovery has been made the emphasis of research has swung in that direction with the same uncontrolled and illogical enthusiasm that a group of children in a zoo might rush from one cage to another as peculiar noises or activity interested them. Time, money and effort can be saved by establishing a more business-like and coordinated control of the use of funds in the field of cancer research. This effort will undoubtedly be made in the near future.*" [Italics added.]

these are valuable traits even when viewed from the standpoint of enlightened selfishness.

All this may seem to some a far cry from researches in chemotherapy of cancer. But, as the Chairman of your Seminar Committee and the Presiding Officer stated, detailed presentations of technical data on chemotherapy experiments are available from the literature and from discussions at other types of meetings; they felt that it would be helpful and stimulating to present today, at this seminar, considerations of a general nature on the relationships among research laboratories and research clinics.

To recapitulate, the clinical side by itself usually does not know which agents are likely to be worth studying in patients, or how to improve agents found to be of limited usefulness; furthermore, systematic large-scale screening of compounds in patients is not feasible. By itself, the laboratory side cannot know whether the chemical agents it develops have the desired effects on tumors in patients and whether they have undesirable side-effects. Their efforts taken together, however, hold forth bright promise of progress.

It is not the function of the chemotherapy laboratory to work out and, at intervals, to announce dramatically the finding of "cures" for cancer. On the other hand, it is not the function of such research laboratories to serve merely as pharmacy shops for clinicians. As I see it, the area of responsibility of the chemotherapy laboratory is: 1) to expand our knowledge about cancer, from the point of view of chemical treatment of tumors, in such a way as *to develop cancer chemotherapy as a basic science;* 2) to forge new chemical agents and new biochemical techniques with the goal of possible clinical usefulness in mind; 3) with the aid of animal tumors to make selections, from among these chemical agents and techniques, of those which might warrant investigation in patients; 4) to collaborate with the clinicians in the follow-through in the clinical studies, with particular attention paid to the drawbacks encountered in the employment of those agents which are found to have some therapeutic effect; and 5) to investigate the mechanism of action of these new drugs upon tumors and upon normal tissues and functions with the aim of overcoming objectionable features and of enhancing potency. The activities should be carried forward in a critical fashion with a calm, tranquil attitude, and with avoidance of frenzied anxiety over quick results (*17, 18*) or over being beaten to the finish line by other investigators.

Researches conducted in this way, with harmonious integration of the work of the chemotherapy laboratory and of the research ward, should gradually extend the frontiers of our knowledge and, in time, in conjunction with surgery and with radiation in its various forms, bring greater relief to the cancer patient.

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METABOLISM OF N^{15}-LABELED *p*-DIMETHYLAMINOAZOBENZENE IN RATS [1]

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Since the first demonstration by Yoshida (*1*), Sasaki and Yoshida (*2*), and Kinosita (*3*) that certain azo dyes produce hepatic tumors in rats, *p*-dimethylaminoazobenzene (DAB) has been the subject of many investigations. An extensive literature (*4*) has been accumulated in an attempt to demonstrate the effect of dietary factors on the incidence of tumors produced by this compound. Furthermore, intensive studies have been made concerning the biologic degradation of DAB.

In more recent years attempts have been made to determine the mechanism of hepatic-tumor formation from biologic degradation studies of DAB and the possible effect on the liver proteins by this compound. Stevenson, Dobriner, and Rhoads (*5*) have demonstrated that demethylation, reduction, and hydroxylation all occur at some stage in the metabolism of DAB by the rat, and, among other products, they have isolated *p*-phenylenediamine. Mueller and Miller (*6*) found that liver homogenates can reductively cleave DAB at its azo group. The presence of *p*-dimethylaminoazobenzene, *p*-monomethylaminoazobenzene and *p*-aminoazobenzene in the tissues of rats fed DAB has been demonstrated by Miller, Miller, and Baumann (*7*), and the existence of a bound azo-dye-protein complex in the liver of rats fed DAB has been conclusively proved by Miller and Miller (*8*).

To date little information is available concerning the quantitative aspects of the fate of DAB in the rat. Boissonnas, Turner, and duVigneaud (*10*) in their studies on the metabolism of DAB labeled with C^{14} in the methyl group, made a quantitative study only of the C^{14} expired as CO_2 and urinary urea. The present investigation was undertaken to obtain quantitative data on the fate of DAB nitrogen in the rat. For this purpose three preparations of DAB (*10*) were used, each labeled with N^{15} in a different position. Data are also given for the distribution and excretion of DAB nitrogen by rats at various intervals during the tumor-forming period.

MATERIALS AND METHODS

Male Osborne-Mendel rats weighing 100-120 gm. were used throughout. Their regular diet consisted of Purina chow supplemented daily with lettuce; the DAB diet, which had the following percentage composition,

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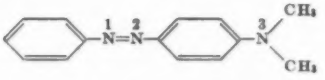
is the one generally used in this laboratory for the routine production of (primary) hepatomas: Casein 6, starch 49.5, Crisco 24.0, sucrose 15.0, cod liver oil 1.0, inorganic salts (*8*) 4.0, dried brewer's yeast 5.0, cystine 0.5, DAB 0.06; the basal diet is the same as above except 0.06 percent starch is substituted for 0.06 percent DAB.

The N^{15}-labeled DAB was generally administered by stomach tube, each rat receiving 6 mg. of the compound (containing 60.5 atom-percent excess of N^{15} in the labeled position) in 0.1 ml. tripropionin. Urine, feces, and various tissues were analyzed for total nitrogen and N^{15} concentration. Two methods were used for the analysis for total nitrogen. When preparations of DAB containing N^{15} in the azo linkage were used, the tissues were first heated with red phosphorus and HI in a sealed tube at 200° C. for 4–6 hours, and this treatment was followed by the usual Kjeldahl procedure after removal of HI. When the preparation of DAB labeled in the N-dimethyl position was used the Kjeldahl procedure alone was found to give complete recovery of the N^{15}. All N^{15} determinations were made with the Consolidated-Nier Mass Spectrometer.

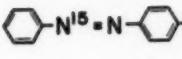
At least four rats were used for each of the values reported. For purposes of simple reference the three preparations of DAB used will be called DAB–1, DAB–2, and DAB–3, and will refer to N^{15} in the position as indicated below.

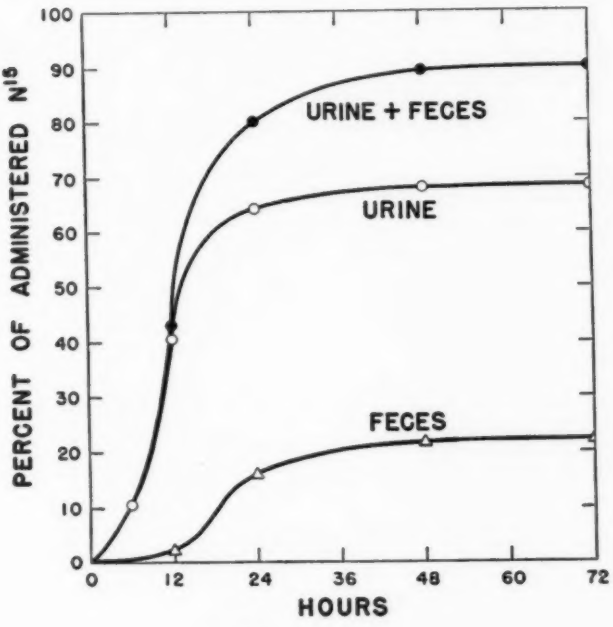


RESULTS

The excretion of DAB-nitrogen following a single dose of N^{15}-labeled DAB to stock rats is indicated in text-figures 1, 2, and 3, which represent the results obtained with DAB–1, DAB–2, and DAB–3, respectively.

Analysis of various tissues 24 and 72 hours after a single dose of N^{15}-labeled DAB to rats showed that negligible amounts of N^{15} were present in tissues such as heart, kidney, blood, lung, spleen, and brain, while significant amounts were found in the gastrointestinal tract and the liver. Data for these latter organs are given in tables 1 and 2. These data show that appreciable though variable amounts (0.05–7.3 percent) of N^{15} remained in the stomach 24 hours after a given dose of isotopic DAB. Appreciable amounts were also found in the large intestine (0.41–6.1 percent), and smaller amounts in the small intestine (0.25–1.8 percent) and the liver (0.81–1.5 percent) (table 1). The amounts of N^{15} in the organs of the gastrointestinal tract were considerably smaller 72 hours after a given dose of N^{15}-labeled DAB (table 2) than after 24 hours; the liver, however, showed no significant change. The average total recovery of administered N^{15} in the urine, feces, gastrointestinal tract, and liver, was 88–93 percent at 24 hours (average of all three nitrogen positions) and 92–98 percent at 72 hours, indicating that essentially all of the administered N^{15} could be accounted for in these regions.

EXCRETION OF N^{15} FOLLOWING ORAL ADMINISTRATION OF  TO RATS



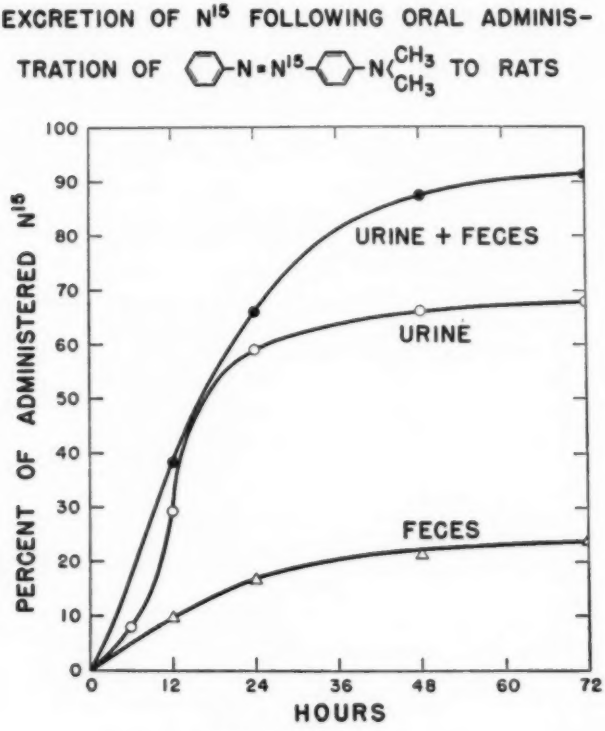
TEXT-FIGURE 1.

TABLE 1.—*Distribution of N^{15} in rats 24 hours after a single oral dose of N^{15}–DAB*

Material	Percent of administered N^{15}		
	[1] DAB-1	[1] DAB-2	[1] DAB-3
Urine	64.3±3.99	59.1±2.01	60.8±2.66
Feces	15.8±4.34	16.9±1.62	16.2±2.73
Stomach and contents	5.0±1.70	7.3±3.58	2.4±1.22
Small intestine and contents	1.1±0.29	1.7±0.13	1.8±0.63
Large intestine and contents	4.4±1.33	3.7±0.54	6.1±0.78
Liver	0.9±0.03	1.5±0.16	1.1±0.0
Total	90.3±2.0	90.6±1.7	89.7±2.1

[1] Mean ± standard error for 4 rats.

To determine the effect of continuous feeding of DAB on the excretion pattern of DAB nitrogen, rats were placed on a diet containing unlabeled DAB and at various intervals were given, by stomach tube, a single dose of 6.0 mg. of isotopic DAB. During the 24 hours following the labeled DAB the animals were fed the basal diet and then the unlabeled DAB diet was given again. The results of this experiment are recorded in table 3,



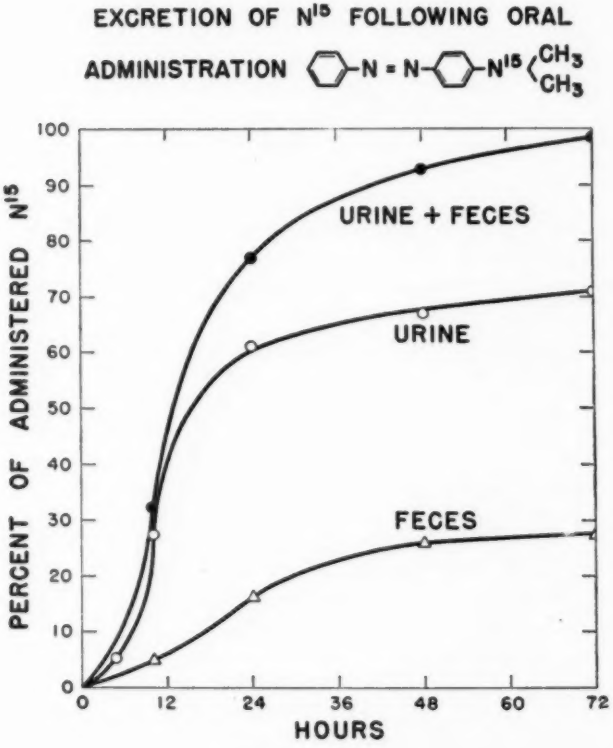
TEXT-FIGURE 2.

TABLE 2.—*Distribution of N^{15} in rats 72 hours after a single dose of N^{15}–DAB*

Material	Percent of administered N^{15}		
	[1] DAB-1	[1] DAB-2	[1] DAB-3
Urine	68. 2±8. 03	67. 7±2. 47	70. 8±2. 30
Feces	21. 8±7. 56	23. 6±2. 13	27. 4±2. 4
Stomach and contents	0. 05±0. 01	0. 2±0. 04	0. 3±0. 03
Small intestine and contents	0. 25±0. 02	0. 3±0. 02	0. 9±0. 18
Large intestine and contents	0. 81±0. 05	0. 4±0. 14	1. 2±0. 39
Liver	0. 81±0. 05	0. 9±0. 06	1. 1±0. 02
Total	91. 7±1. 1	92. 8±3. 0	102. 0±4. 0

[1] Mean ± standard error for 4 rats.

which shows that continuous feeding of DAB over a 4-month period did not alter the general excretion pattern of DAB nitrogen. The average urinary excretion of DAB nitrogen during this period was greater than the



TEXT-FIGURE 3.

fecal excretion by a ratio of 2.5/1 to 3.4/1. The average 72-hour total excretion of N^{15} during this period was 89 percent or more of the administered DAB nitrogen.

TABLE 3.—*Effect of feeding DAB on the excretion pattern of DAB–N (72 hours)*

Time on unlabeled DAB	Percent of administered N^{15}					
	Urine			Feces		
	[1] DAB-1	[1] DAB-2	[1] DAB-3	[1] DAB-1	[1] DAB-2	[1] DAB-3
0	68. 2	67. 7	70. 8	22. 6	24. 0	28. 6
7	74. 1	66. 1	65. 8	17. 7	23. 8	28. 6
30	72. 3	73. 4	60. 9	16. 2	16. 1	31. 1
120	63. 3	81. 3	71. 5	25. 1	19. 8	18. 3
Average	69. 5	72. 1	67. 3	20. 4	20. 9	26. 7

[1] Mean value for 4 rats in each group.

TABLE 4.—*Effect of feeding unlabeled DAB on the uptake of N^{15} by the liver following a single dose of N^{15}–DAB*

Days on unlabeled DAB	Atom percent excess $N^{15} \times 10^{-3}$					
	24 hours			72 hours		
	DAB-1	DAB-2	DAB-3	DAB-1	DAB-2	DAB-3
Control [1]	1. 2±. 02	1. 6±. 02	1. 4±. 05	1. 0±. 04	1. 2±. 07	1. 3±. 03
7 [2]	1. 4±. 12	2. 5±. 02	2. 9±. 46	0. 9±. 07	1. 1±. 12	1. 1±. 18
30 [2]	2. 3±. 26	3. 9±. 25	3. 0±. 29	1. 5±. 13	1. 6±. 16	3. 0±. 48
120 [2]	2. 3±. 01	3. 8±. 04	4. 1±. 49	1. 3±. 05	1. 8±. 38	2. 5±. 02

[1] Mean ± standard error for 8 rats.
[2] Mean ± standard error for 4 rats.

The amount of N^{15} in the liver following a single dose of N^{15}-labeled DAB was small (table 1), being somewhat larger when DAB–2 and DAB–3 were given than when DAB–1 was given. When rats were fed unlabeled DAB for 1 week and then given a single dose of N^{15}-labeled DAB, the N^{15} concentration in the liver 24 hours after the administration of the isotopic compound was greater than when the isotopic DAB was given as an initial dose (table 4). This effect was greater, for DAB–1 and DAB–2, after 1 month on unlabeled DAB than after 1 week, and was approximately the same at 1 month and 4 months.

Prolonged feeding of isotopic DAB resulted in a much higher N^{15} concentration in the liver than was obtained with a single dose (table 5) and gives a truer picture of the continual state of this organ. It can also be seen from the data in table 5 that the N^{15} of DAB-2 and DAB-3 is retained to a greater extent than that of DAB-1 and indicates a difference in the rate of metabolism of the two rings of DAB. None of the rats used in this experiment had hepatic tumors at 120 days. The livers were pale and appeared "precancerous."

DISCUSSION

The very great excretion of DAB nitrogen and the very small amount found in the liver may, in part, explain the necessity for continuous feeding of this compound for the production of tumors. This is further indicated by the fact that the excretion of DAB-nitrogen continues to be high for several months, implying perhaps that a large excess of the compound must be present at all times in order to push the reactions involved in the tumor-producing process.

TABLE 5.—*Effect of feeding N^{15}-DAB on concentration of N^{15} in the liver*

Days on unlabeled DAB	Days on labeled DAB	N^{15} Concentration Atom percent excess × 10^{-3}		
		[1] DAB-1	[1] DAB-2	[1] DAB-3
Control	1	1. 2±. 02	1. 6±. 02	1. 4±. 05
30	1	2. 3±. 26	3. 9±. 25	3. 0±. 29
	10	4. 3±. 38	6. 9±. 58	6. 5±. 80
120	1	2. 3±. 01	3. 8±. 04	4. 1±. 49
	10	3. 4±. 37	4. 9±. 05	4. 8±. 07

[1] Mean ± standard error for 4 rats.

The small amount of DAB nitrogen present in the liver indicates that only a small fraction of the DAB (or derivatives thereof) is intimately associated with the tumor-producing process. Miller and Miller (*8*) have established the existence of a bound azo-dye-protein complex in the livers of rats fed DAB, which they have shown reaches a maximum in 4 to 6 weeks. The significance of this complex in the tumor-forming process has, as yet, not been completely answered. The present data (table 4) show that the uptake of DAB nitrogen by the liver following a single dose of N^{15}-labeled DAB is greater when rats have been fed a DAB-containing diet for 1 month than for 1 week, or 1 day, while prolonged feeding of N^{15}-labeled DAB (table 5) resulted in a maximum N^{15} concentration in the liver at about 5 weeks. Of special interest is the greater retention of the N^{15} of DAB-2 and DAB-3 as compared to DAB-1 (table 5). This suggests that in the degradation of DAB in the organism the fractions derived from the diamine half of the molecule are held more firmly, in the liver, than those of the aniline half of the molecule. Miller *et al.* (*9*) suggested that the link in the bound azo-dye-protein complex is through the N-dimethyl group or the ring to which it belongs. The present data (table 5) show that this ring is retained to a greater extent by the liver but not as azo dye alone. They indicate the retention of fragments of DAB, whose nature may be of importance to the tumor-forming process.

Stevenson *et al.* (*5*) have demonstrated that *p*-phenylenediamine may be intermediate in the metabolism of DAB. The possibility does exist that the bound azo-dye-protein complex first appears in the liver, which then undergoes cleavage at the azo linkage to produce a *p*-phenylenediamine-protein complex, which in turn may be the hepatoma-forming nucleus. Indirect arguments against this possibility lie in the fact that neither *p*-dimethylaminoaniline nor *p*-phenylenediamine produces hepatic tumors when administered orally (*4*). The metabolism of N^{15}-labeled *p*-phenylenediamine is now under investigation.

SUMMARY

Ninety percent or more of the N^{15} of a given dose of isotopic DAB was excreted by Osborne-Mendel rats in 72 hours. Approximately 70 percent of the administered N^{15} was excreted in the urine and 20 percent in the feces. This was true for each of the three nitrogens of DAB. The excretion pattern of DAB-nitrogen was not appreciably affected by continuous feeding of DAB over a period of 4 months.

The amount of DAB nitrogen in the liver is very small, and is greater at 5 or 6 weeks than at 4 months or 1 day.

The nitrogen of the methyl substituted aniline ring of DAB is retained by the liver to a greater extent than that of the unsubstituted ring.

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HISTOLOGY AND CYTOCHEMISTRY OF HUMAN SKIN. I. METACHROMASIA IN THE MONS PUBIS [1] [2]

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INTRODUCTION

In the skin of the mons pubis, growing hair follicles stain metachromatically. Metachromasia is also observed in the fibers and some fibroblasts of the loose connective tissue around sebaceous glands and capillary beds in the upper parts of the dermis. The metachromasia here described is shown best when sections are stained in toluidin blue buffered to pH 5.0 or 6.0, although in the papillae of growing hairs it is shown even at pH 2.3. Since it resists extraction in 96 percent alcohol, it must be considered true metachromasia (*1*).

Human skin (*2*) and the skin of the pig and calf (*3*) yield appreciable quantities of hyaluronic acid and chondroitin sulfate by chemical analysis. Since toluidin blue stains acid polysaccharides metachromatically in tissue sections (*1*, *4–9*), an analysis of metachromasia should give some indication of the distribution of the chondroitin sulfates, and possibly of hyaluronic acid, if this latter substance is present in sufficient amounts (*9*).

MATERIALS AND METHODS

Skin from 11 men (18 to 38 years of age) was excised under general anesthesia from the mons pubis. Tissues were fixed in Helly's fluid, embedded in paraffin, and sectioned at 5 μ. They were stained in 0.05 percent toluidin blue buffered with McIlvaine buffer to pH 2.3, 3.0, 4.0, 5.0, 6.0, 7.0, and 8.0. Some sections were stained in each of the buffered solutions of toluidin blue for 5 minutes; they were quickly rinsed in water, blotted dry around the tissue, but leaving the section moist, and placed 5 minutes each in 2 changes of tertiary butyl alcohol. They were then placed 2 minutes in absolute ethyl alcohol, cleared in toluol, and mounted in piccolyte dissolved in toluol. Sections so treated show a remarkable uniformity in the intensity of the staining. Long rinses in ethyl alcohol must be avoided, because ethyl alcohol removes much of the orthochromatic dye but not the metachromasia. After staining with toluidin blue some sections were left 10 minutes in 96 percent ethyl alcohol, which is

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said to remove false metachromasia (*12*). Before staining in toluidin blue, sample sections were incubated 3 hours at 60° C. in 0.001 percent ribonuclease buffered to pH 6.7 with McIlvaine buffer (*10*). Control sections were maintained at 60° C. in the buffer alone. Other sections were incubated 18 hours at 37° C. in an equivalent of 15 turbidity units of testicular hyaluronidase dissolved in each milliliter of 0.3 percent NaCl (*11*), before they were stained in toluidin blue. Control sections were maintained at 37° C. in the salt solution without the enzyme.

OBSERVATIONS

In the skin of the mons pubis there is abundant metachromasia in hair follicles that contain growing hairs (figs. 1 and 2). The ground substance of the papilla, which in resting hairs stains orthochromatically, in growing hairs is intensely metachromatic, even when sections are stained at pH 2.3. The cytoplasm of the papilla cells and of the capillary endothelial cells stains orthochromatically (fig. 2). At its base, the papilla is connected with the connective-tissue sheath of the hair follicle by a narrow band of tissue, which is not metachromatic (fig. 1). The mass of connective tissue beneath the lips of the bulb is lightly metachromatic, as is also the connective-tissue sheath around the lower third of the follicle (fig. 3). The connective-tissue sheath becomes more strongly metachromatic around the middle third of the follicle, and this metachromasia may extend to the level of the sebaceous glands. The connective tissue that surrounds hair follicles and sebaceous glands is composed of delicate, metachromatic fibers, the metachromasia being first detected at pH 5.0. The fibroblasts in this tissue are usually orthochromatic but some larger ones stain metachromatically.

Around the base of the hair bulb, the cells of the single-layered external sheath show diffuse cytoplasmic metachromasia (fig. 1). As the external sheath becomes stratified in the upper part of the lower third of the follicle, most of its cells, particularly those in contact with the glassy membrane, contain numerous metachromatic granules (fig. 4). The metachromatic granules in these cells are found together with a moderate amount of glycogen (*15*). The cells of the middle third of the external sheath, which are laden with glycogen (*15*), contain only a few metachromatic granules. In the external sheath, metachromasia is detectable at pH 2.3 and 3.0, and it becomes progressively intense from pH 4.0 to 8.0. The matrix cells immediately around the metachromatic papilla show slight metachromasia at pH 5.0 to 8.0, but these cells are strongly basophilic, and the orthochromatic staining partially masks the metachromasia (figs. 1 and 2, *cf. 12*).

The cytoplasm of the secretory cells of the eccrine glands at pH 3.0 stains a lilac color, which is intensified at pH 4.0 and 5.0. At pH 5.0 the apices of the secretory cells stain a light purple, suggesting a mixture of metachromatic and orthochromatic staining. At higher pH levels the cells stain so intensely that metachromasia is masked. This, then, is not comparable to the findings of Lison and Mutsaars (*13*)—that ribonucleic

acids may stain metachromatically in tissues stained in strong solutions of toluidin blue. The apical, dense basophilia corresponds to the tab of periodic acid-Schiff reactive diastase-resistant material described by Bunting *et al.* (*14*) and by Montagna *et al.* (*15*). The staining of the apocrine glands is similar to that of the eccrine glands. There is no metachromasia in the ducts of either type of gland.

In the dermis, the recognizable mast cells contain strongly metachromatic granules from pH 2.3 to 8.0. Some of the fibroblasts in the areolar tissue that surrounds the capillary beds and the sebaceous glands, and those in the papillary layer, stain metachromatically at pH 4.0. Some of these fibroblasts contain variable numbers of metachromatic granules and resemble small mast cells. The endothelial cells stain orthochromatically. Elsewhere in the dermis, the fibroblasts assume a weak orthochromatic stain at pH 4.0. The delicate collagenous fibers in the tissue just described attain a light metachromasia at pH 5.0 and it becomes stronger at pH 6.0 to 8.0. It is not possible to ascertain at the present time if these fibers are intrinsically metachromatic, or if the metachromasia represents substances that have diffused away from surrounding cells. The coarser collagenous fibers of the dermis, which in more acid stains remain unstained, stain a light blue at pH 5.0, and dark blue-green at pH 6.0 to 8.0. In the deeper dermis, the areolar tissue around blood vessels and that around sweat glands is seldom metachromatic.

In the surface epidermis, the cytoplasm of the cells in the stratum germinativum is intensely basophilic, but shows no metachromasia. There is a decrease in cytoplasmic basophilia in the cells of the stratum granulosum. The keratohyaline granules stain a deep blue.

Metachromasia is virtually unchanged in sections that have been incubated in ribonuclease before staining at pH 2.3 to 5.0, but cytoplasmic basophilia is abolished in the epidermal cells, and in the cells of hair follicles and sweat glands. When stained at pH 6.0 to 8.0, however, there is always some residual basophilia in these cells. This is not surprising, since the formaldehyde in Helly's fluid may have increased the affinity of proteins for basic dyes. Control sections kept in the buffer alone show no diminution of basophilia or metachromasia. The mast cells, which in control sections are dark purple, are cherry red in sections incubated in ribonuclease. This might indicate either that the mast cells contain ribonucleic acid in addition to heparin or heparin precursors (*16*), or that some of the components of the mast granules have dissolved out.

In sections incubated in hyaluronidase, there is a diminution in the cytoplasmic basophilia of the cells of cutaneous appendages, and there is an abolition of metachromasia in the delicate connective tissue around the hair follicles, sebaceous glands, and capillary beds. Metachromasia remains unchanged in the papilla and external sheath of hair follicles; the mast granules, as in sections incubated in ribonuclease, are stained cherry red. Control sections maintained in the salt solution alone are identical with those incubated in hyaluronidase.

DISCUSSION

Evidence accumulated (*1, 4, 7, 8, 9, 17, 18*) shows that metachromasia, in the main, indicates the presence of acid polysaccharides.

Erratic results obtained with toluidin blue, and the indiscriminate appearance of "false" metachromasia have been eliminated in our laboratory by the use of diluted solutions of toluidin blue buffered from pH 2.3 to 8.0. The metachromasia described here is revealed at acid pH values; it resists alcoholic extraction, and must be considered "true" metachromasia (*1, 4*). Although Sylvén (*12*) asserts that "the usual fixation methods involve an almost complete loss of metachromatic material from the cytoplasm of hair epithelia" and that only frozen-dried material can be used effectively, it would be difficult to imagine more brilliant metachromasia than that seen in the hair papilla and in the external sheath in skin properly fixed in Helly's fluid.

Normal human skin contains appreciable amounts of mucopolysaccharide—hyaluronic acid and chondroitin sulfate (*2*). The skin of the pig and calf (*3, 19*) also contains hyaluronic acid together with chondroitin sulfate B, a substance not digested by pneumococcal or testicular hyaluronidase. Although some authors believe that both hyaluronic acid and chondroitin sulfate stain metachromatically (*11, 17, 18*), Hale (*8*) states that "hyaluronic acid and similar polysaccharides, which probably contain no sulfate * * * do not stain metachromatically." Furthermore, Meyer (*9*) finds that while 1-percent concentrations of hyaluronates stain metachromatically, weaker concentrations do not. Since the concentrations of hyaluronates in tissue are probably not this high, metachromasia in tissues may indicate only sulfate-containing compounds, largely chondroitin sulfate.

Concerning the significance of metachromasia in growing hair follicles, Sylvén (*12*) states that it indicates polysaccharide-ester sulfates, which "play a role for the supply of sulfurous constituents in keratin synthesis, and should accordingly be regarded as labile S-bearing compounds." In the skin of two hemophiliacs, Sjölin (*20*) finds abundant, diffuse metachromasia in the dermal connective tissue and an increase in the number and size of mast cells. Sjölin believes that this metachromasia may reveal the presence of anticoagulant substances.

Since hyaluronic acid is digested by testicular or streptococcal hyaluronidase whereas chondroitin sulfate B is not (*3*), even if both substances are colored metachromatically, it should be possible to identify them selectively by enzyme hydrolysis. In the skin, metachromasia in the hair papilla, external sheath, and in mast cells resists digestion with testicular hyaluronidase; the weaker metachromasia elsewhere in the skin is abolished. Since it is abolished also in control sections maintained in the salt solution alone, it may have been washed away rather than hydrolyzed by the enzyme. In any case, the evidence strongly suggests that the metachromasia of the hair papilla and external sheath is due to the presence of chondroitin sulfate B.

SUMMARY

For the demonstration of metachromasia the toluidin-blue technique has been standardized by buffering 0.05 percent solutions of the dye to pH 2.3, 3.0, 4.0, 5.0, 6.0, 7.0, and 8.0. Sections stained at known pH levels show a remarkable uniformity.

In normal skin, the papillae of growing hair follicles are intensely metachromatic, as are also the cells of the external sheath in the upper part of the lower third of the follicle. The connective-tissue sheath usually shows some metachromasia. Metachromasia is also found in the delicate connective tissue around sebaceous glands and capillary beds in the dermis.

Metachromasia here described is visualized at acid pH and resists extraction in alcohol. The metachromasia in the hair papilla and in the external sheath is not abolished by testicular hyaluronidase, and may represent chondroitin sulfate B.

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PLATE 62*

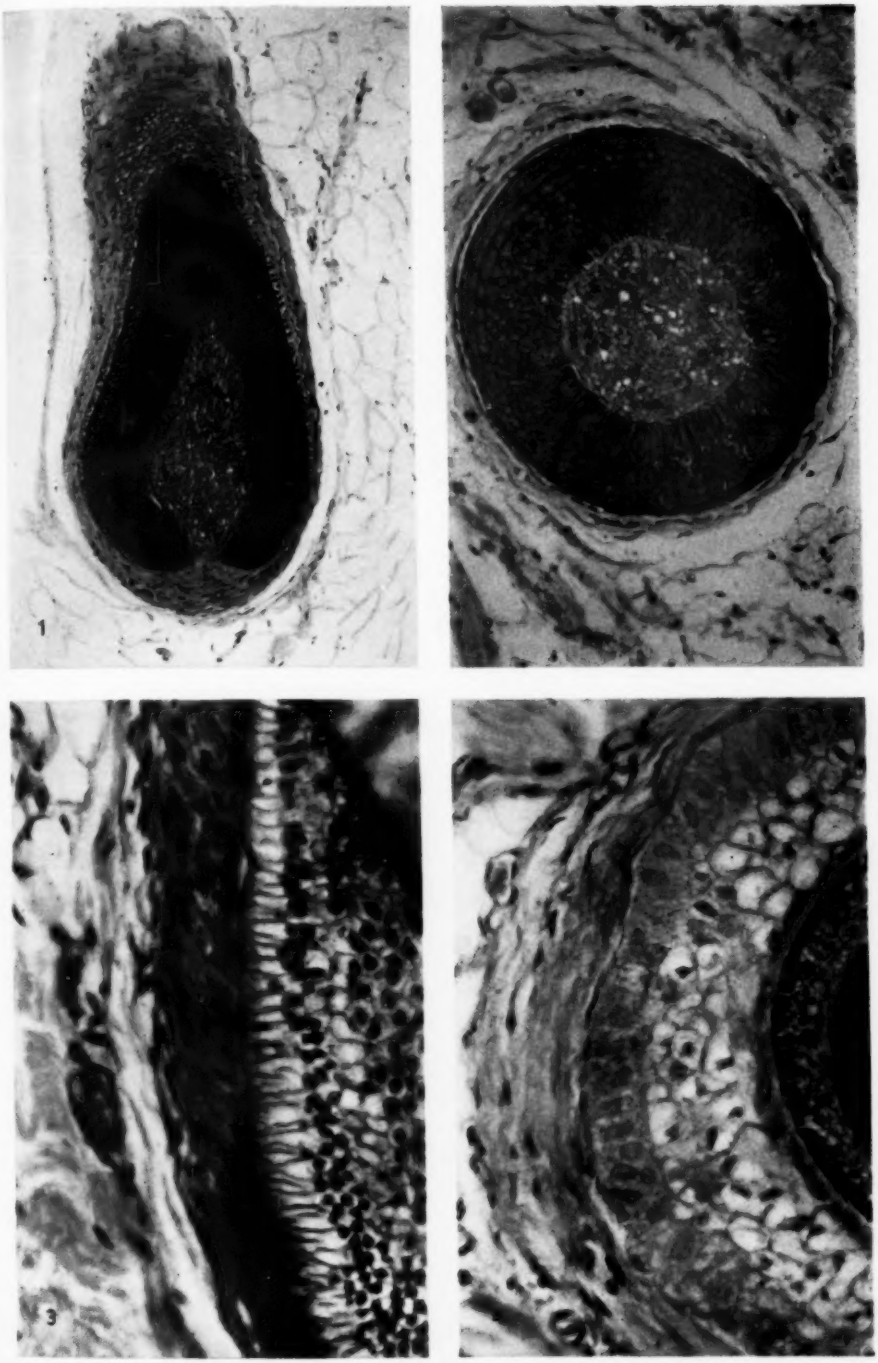
FIGURE 1.—Longitudinal section through the bulb of an actively growing hair follicle, stained in toluidin blue buffered to pH 6.0. Brilliant metachromasia in the hair papilla and in the external root sheath. Delicate metachromasia in the connective-tissue sheath. × 90.

FIGURE 2.—Transverse section of the bulb of a growing hair follicle, stained at pH 5.0. Metachromasia is conspicuous in the papilla. Metachromasia in the matrix cells is partially masked by their intense basophilia. The delicate connective tissue surrounding the follicle shows some metachromasia. × 200.

FIGURE 3.—Metachromasia in the connective-tissue sheath of the middle third of an active hair follicle stained at pH 5.0. The clear columnar cells of the external sheath were laden with glycogen. × 300.

FIGURE 4.—Massive metachromasia in the cells of the external sheath, stained at pH 6.0. The vacuolated cells contained glycogen. Note delicate metachromasia in the connective-tissue sheath. × 300.

*The colored photographs were taken by the photographic staff of the Department of Anatomy, Harvard Medical School. We are deeply indebted to Dr. George B. Wislocki for making this possible.



Montagna *et al.*

MYELOGENOUS LEUKEMIA IN THE CAT [1]

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Myelogenous leukemia is infrequently diagnosed in cats. It has been reported by Ball (*1*), Lund (*2*), Julliard (*3*), Lillie (*4*), and Bloom (*5*). Ball and Julliard merely referred to the occurrence of this disease in cats and gave no details of its nature. Bloom stated that he had observed several cases of basophilic myelocyte leukemia in the cat. Lund diagnosed his case as "myelogenous neutrophil and eosinophil leukemia" and noted enlarged spleen, slightly enlarged lymph nodes and gray-yellow bone marrow associated with it. Lillie's case of "mast myelocyte leukemia" was not recognized as such at autopsy, and only the liver, kidney, and spleen were presented to him for histologic examination. He described mast myelocyte infiltration of the splenic pulp and liver capillaries, and mast myelocyte nodules in the liver. Of these reports, only the cases of Lund and Lillie can be accepted on the basis of descriptive evidence presented.

Lymphatic leukemia, on the other hand, has been observed more frequently in the cat [Siedamgrotzky (*6*), Didier (*7*), Pinvidic (*8*), Lund (*2*), Ball and Auger (*9*), and Bloom (*10*)]. Other cases that might be considered in this group are those of Joest (*11*), who described a case of splenic leukemia in the cat; deDoës (*12*), pseudoleukemia; and Lellmann (*13*), who reported a mixed leukemia which, as suggested by Lillie, may have been a hyperregenerative megaloblastic anemia rather than leukemia. Sommer (*14*) discovered two cases of leukemia in cats, but the type was not specified.

The reported occurrence of types of leukemia in cats follows the general pattern in other species of domestic animals. Granulocytic leukemia and myeloma have been reported in horses, dogs, chickens, pigs, and cattle, however; with the exception of the chicken a true leukemia with malignant cells in the peripheral blood is seldom observed. A condition resembling chloroma in human beings is occasionally seen in these species (*15*). Lymphocytic leukemia and lymphosarcoma are seen with relatively greater frequency in domestic animals than are the granulocytic types.

The present case of myeloid leukemia in a cat was diagnosed clinically 4 days before death, and followed by post-mortem examination. Leukemic cells were studied by phase contrast microscopy and transplanted into the anterior chamber of the eye of five normal cats.

[1] Received for publication September 17, 1951.

[2] National Institutes of Health, Public Health Service, Federal Security Agency.

CASE REPORT

History

A 7-year-old male tabby cat was presented for examination, with the explanation by its owner that it had been listless and losing weight over a period of 6 weeks. Another cat, belonging to the same owner, appeared healthy. There was no history of exposure to irradiation or of intoxication with benzol, aniline dyes, or related substances. No information could be obtained regarding the heritage of the cat or its siblings.

At the time of initial examination, breathing was labored, the mucous membranes were pale, and the cat had a glassy stare. However, the pupils reacted normally to light and accommodation. No lymph nodes were palpable. The temperature was normal. Repeated examination of the blood was made and the data are reported in table 1.

Examination of the Blood

Red blood cells were stained supravitally for Heinz bodies according to the technique described by Webster (*16*), and the proportionate number of cells containing these bodies was determined. Counts of cells containing Heinz bodies were also done with the phase microscope. These bodies were present in 43 percent of the circulating red blood cells. Weekly counts on five normal adult cats showed a range of 1 to 11 percent red blood cells containing Heinz bodies. Repeated erythrocyte counts from the leukemic cat, as shown in table 1, indicated marked anemia. Numerous immature cells of the erythrocyte series were seen in the blood smears.

Hemoglobin determinations were made with the Coleman Junior spectrophotometer. Results are recorded in table 1. The final reading, taken the day before death, was less than 2.0 gm. per 100 cc. A single platelet count, taken 3 days prior to death, was found to be 67,000 per cu. mm. The normal range for platelets in cats is given as 200,000 to 600,000, with an average of 422,000 (*18*).

The total leukocyte count ranged from 43,100 to 59,950. Such variations are described during the course of leukemia in man. Examination of the blood films showed an increase in intermediate forms of myeloid blood cells in addition to large numbers of primitive "stem-cell" types. These latter cells were nearly twice as large as adult neutrophils and had a basophilic cytoplasm containing occasional basophilic granules. The nuclei were correspondingly large, hyperchromatic, and contained from 1 to 4 nucleoli (fig. 8). Occasionally, these large cells possessed indented nuclei with neutrophilic granules in the cytoplasm. Auer bodies were not seen. Many smudge cells were present, indicating the fragility of these cells. The peroxidase reaction was not studied.

Several mitotic figures were seen in smears of peripheral blood and in fresh blood examined at room temperature under the phase microscope (figs. 6 and 7). Three cells in mitosis were followed to late telophase or to complete division. One of the mitotically dividing cells was observed

TABLE 1.—*Circulating blood cells of myelogenous leukemic cat compared with blood of normal animal*

Date	Red-blood-cell count	Hemoglobin gm. 100cc.	White-blood-cell count total	Segmented	Promyelocyte	Myelocytes	Myeloblasts	Lymphocytes	Eosinophils	Basophils	Monocytes	Nucleated red blood cells
Blood cells of myelogenous leukemic cat												
Oct. 13	2, 140, 000	2. 4	43, 100	5	2	14	34	45	0	0	?	6
Oct. 14	1, 440, 000	2. 0	50, 500	10	10	9	38	33	0	0	?	8
Oct. 17	1, 529, 000	less than 2. 0	59, 950	8	8	16	48	20	0	0	?	14
Blood cells of normal adult cat [1]												
Average	7, 656, 000	11. 53	15, 874	52. 35	------	7. 1	0	33. 96	5. 5	0	1. 55	0
Range	4. 06–11. 29	8. 7–14. 55	5, 000–46, 400	34. 4–66. 9	------	1. 7–13. 5	0	24. 9–48. 5	4. 3–6. 9	0	0. 8–2. 3	0

[1] Taken from Gardner, M. V.: "The blood picture of normal laboratory animals," Journal of The Franklin Institute, Vol. 243, No. 1, January 1947 (*17*).

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from prophase through the completed cycle, a process which required a little less than 30 minutes to complete. The occurrence of mitoses in the peripheral blood of human patients with acute myelogenous leukemia has been described by Willis (*19*).

The animal survived in the laboratory for 4 days. On the fourth day it appeared moribund and was given a lethal dose of ether. A post-mortem examination, including the central nervous system, was performed. Blocks of tissue were fixed in Zenker-formol fluid and in 10 percent formalin. Sections from all the organs studied were stained with hematoxylin and eosin, toluidin blue, the Prussian blue reaction for iron, the Giemsa stain, and the periodic-acid-Schiff reaction. Kidney sections were also stained with Sudan III.

Gross Pathology

The liver weighed 190 gm. and appeared slightly larger than normal. Its external surface showed numerous dark red streaks which on section were found to extend throughout the parenchyma, apparently outlining the perimeter of lobules. The spleen weighed 30 gm. and appeared to be normal in size, color, and consistency. The lymph nodes were normal in appearance and size, with the exception of those of the periaortic group, which appeared enlarged, one of these nodes measuring 2 cm. in its greatest dimension. The bone marrow of the sternum, ribs, calvarium, and femora were dark red and gelatinous. The femoral marrow had the consistency of firm jelly and retained its shape following removal from the cavity in a single piece. Decalcification of this marrow was not necessary, since the trabecular bone had been almost completely obliterated.

No changes in other body organs or tissues were detected.

Histopathology

Liver.—Leukemic infiltration occupied nearly one-half of the liver substance. There was no definite pattern of distribution, although many of the cells were in the sinusoids, and some of the larger accumulations were located in the region of portal triads (fig. 1). There were no discrete solitary nodules. The liver cords were moderately atrophied in areas where the sinusoids were filled with leukemic cells (fig. 2). Some of the hepatic cells contained numerous brown cytoplasmic granules that gave a positive reaction for iron.

Lymph nodes and tonsils.—Lymph nodes, taken from several locations, all showed active follicles, and leukemic cells were present in the sinusoids (figs. 3 and 4). The periaortic nodes showed the largest follicles and the largest numbers of leukemic cells in the sinusoids. There was no evidence of capsular invasion. The tonsillar lymphoid tissues also showed follicular hyperplasia.

Spleen.—The architecture of this organ was not altered. The lymphoid follicles were active, and the sinusoids contained leukemic cells, many of which were in mitosis. The leukemic cells in the sinusoids were confined to the vascular channels. There was no evidence of invasion into the capsule or trabeculae. There was no evidence of myeloid metaplasia.

Bone marrow.—The femoral marrow was completely replaced by a mass of closely-packed undifferentiated cells (fig. 5). Mitotic figures were very numerous, there being as many as nine per high-power field. There were some very small areas of erythropoiesis. A resemblance to the bone marrow of human beings with "stem-cell" leukemia was noted.

Kidney.—The cytoplasm of the epithelial cells lining the convoluted tubules contained many sudanophilic droplets, as is normally seen in the cat. In scattered areas of the renal cortex there were small accumulations of leukemic cells lying free in the supporting connective tissue between the tubules.

Other tissues examined microscopically included the heart, lungs, salivary gland, urinary bladder, gall bladder, small and large intestine, cerebrum, cerebellum, medulla oblongata, pancreas, and tongue. No pathologic changes were seen in these tissues.

HOMOLOGOUS INTRAOCULAR TRANSPLANTS

Small pieces of the swollen periaortic lymph nodes were inserted with a trocar into the anterior chamber of the eye of five normal cats. The transplants regressed rapidly and had disappeared within a period of 1 month. At the end of the 6-month period, these animals were autopsied, and no evidence of leukemia was found.

DISCUSSION

A diagnosis of leukemia was made on examination of the first blood film; however, the primitive cell types present did not have sufficient numbers of "differentiating company" to allow an unequivocal diagnosis as to the cell of origin. It is true that the large number of circulating lymphocytes suggested lymphocytic leukemia, and that the presence of immature myeloid and erythroid cells could have been explained as a disturbance in the bone marrow resulting from invasion by neoplastic lymphocytes. However, microscopic examination of the lymph nodes and spleen showed leukemic cells in the vascular channels only, without vessel-wall or trabecular invasion. Although there was evidence of rather marked hyperplasia in the germinal follicles, the normal architecture was maintained. The bone marrow of the femurs, sternum, ribs, and calvarium was abnormal, consisting of neoplastic cells only. It closely resembled the marrow in human cases diagnosed as stem-cell leukemia. It is believed that the high lymphocyte count in the peripheral blood and the hyperplasia in the germinal follicles of the lymph nodes can be accounted for as a lymphoid reaction to the myeloid disease.

Numerous mitotic figures were found among the leukemic cells of the peripheral blood, and the process of mitosis could be followed under the phase microscope. This demonstrated that in the circulating blood, the leukemic cells were capable of at least completing mitosis, which may have been initiated in the bone marrow, or possibly, of carrying out the entire process in the peripheral blood. In one instance, the time required for division of a cell discovered in the early stages of mitosis, was deter-

mined directly by observation under the phase microscope to be about 30 minutes. The presence of large numbers of immature cells in the peripheral blood may represent a terminal outpouring of cells. Willis (*19*) has referred to the frequent finding of mitotic figures in peripheral blood films of human beings with acute myelogenous leukemia.

Heinz bodies on red blood cells were greatly increased over the normal. This phenomenon has been observed in cats and in other animals following the injection of various organic compounds, such as phenylhydrazine and various aniline dyes. It has also been reported in one case of myelogenous leukemia in man (*20*). The occurrence of these bodies on red cells would appear to be the result of altered metabolism rather than being directly associated with the leukemic process.

Splenic enlargement appears to have been a rather constant feature of the few cases of leukemia in the cat reported in the literature and nearly always occurs in man with this disease. It is suggested that the case reported herein was very acute, and that the extramyeloid potentialities of the spleen did not have sufficient time to develop.

In contrast to some human cases of leukemia, hemorrhage into the tissues and body cavities was not seen.

On the basis of these findings, the diagnosis of myelogenous stem-cell leukemia seems justified.

SUMMARY

A case of myelogenous "stem-cell" leukemia in the cat is described. The clinical diagnosis was made 4 days prior to death, and blood studies were performed. Numerous mitoses were observed in the peripheral blood films and in fresh blood examined under the phase microscope. Leukemic infiltrations were present only in the liver, kidney, spleen, and lymph nodes.

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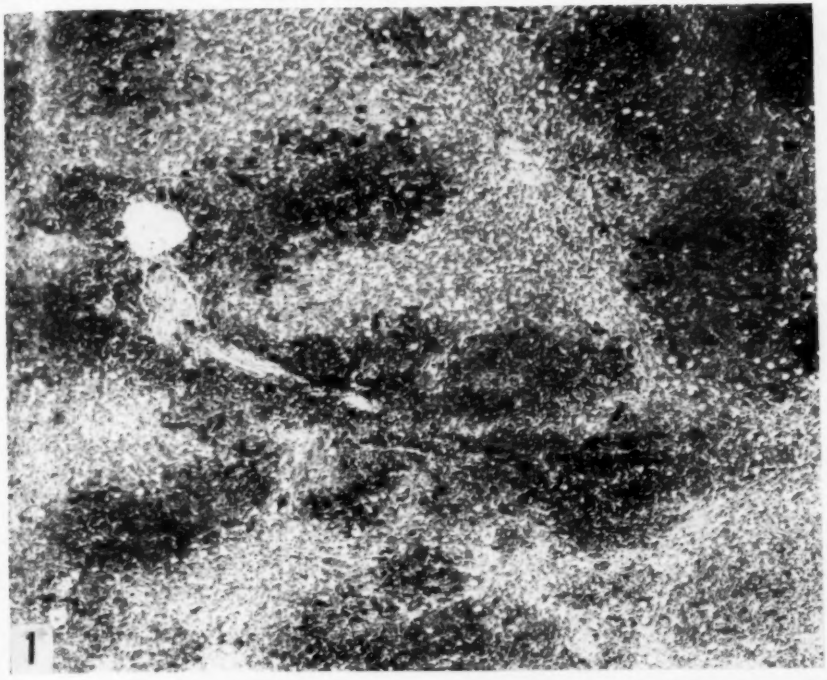
PLATE 63

FIGURE 1.—Liver. Leukemic cells replace nearly one-half of liver tissue. Many of the larger accumulations are located around hepatic triads. Hematoxylin and eosin. × 50.

FIGURE 2.—Liver. Leukemic cells in the sinusoids. Mitotic figures are numerous. Hematoxylin and eosin. × 1,500.



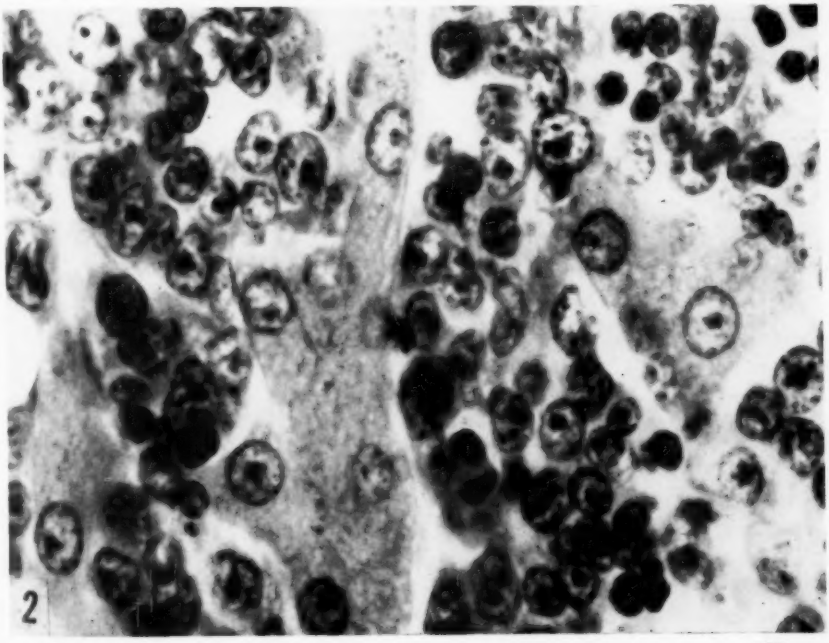


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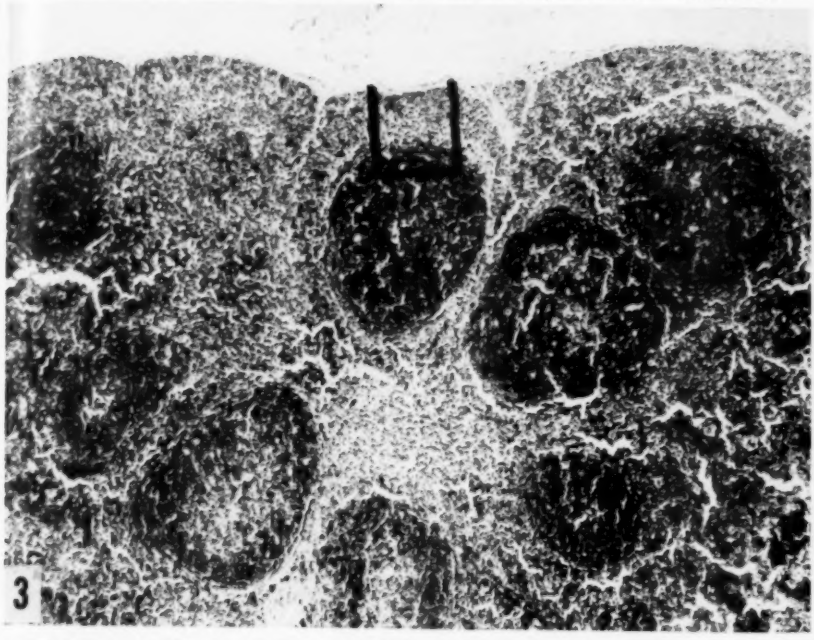
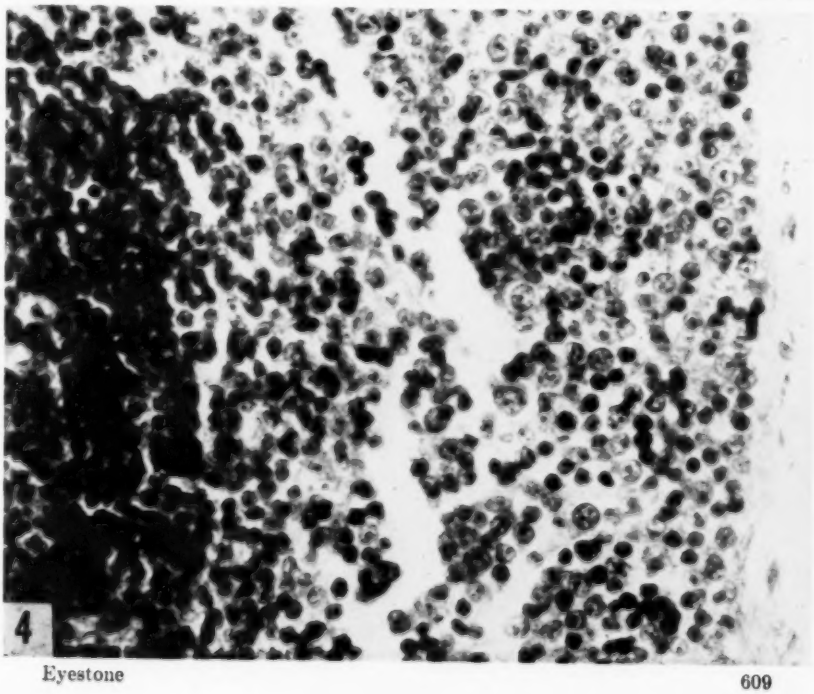
PLATE 64

FIGURE 3.—Periaortic lymph node showing enlarged follicles. The sinusoids are filled with leukemic cells. Hematoxylin and eosin. × 100.

FIGURE 4.—Periaortic lymph nodes. High-power view taken from marked area in figure 3, showing peripheral sinusoids filled with leukemic cells. Hematoxylin and eosin. × 610.



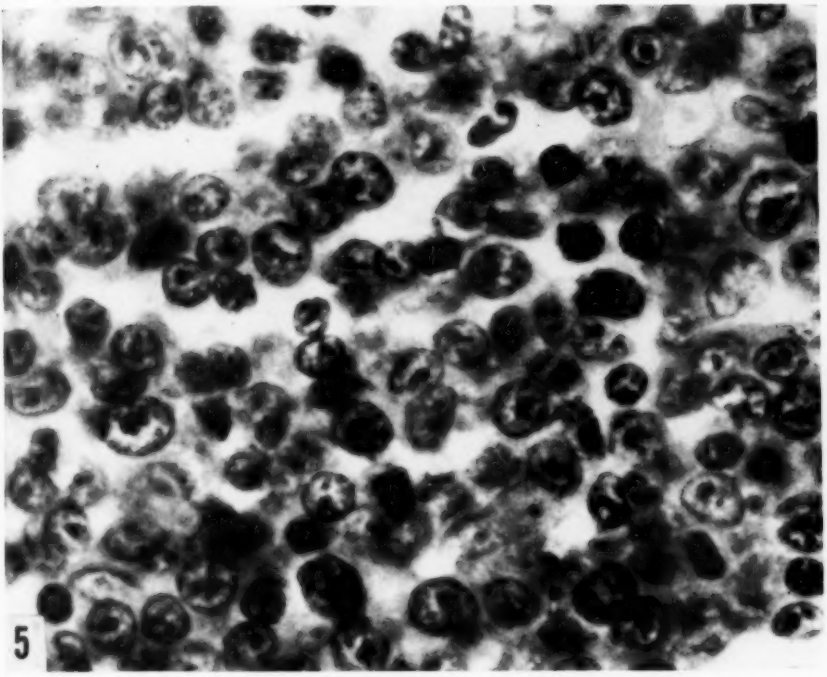


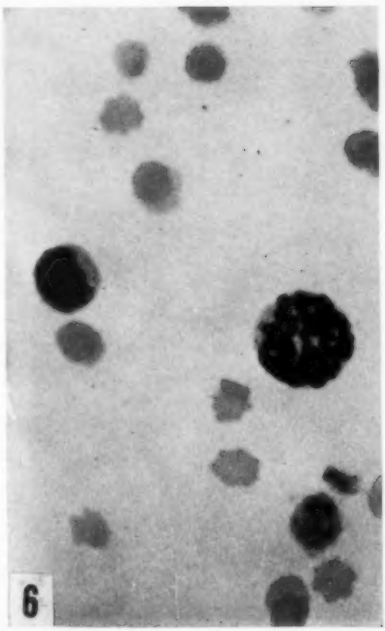
Eyestone

PLATE 65

FIGURE 5.—Femoral bone marrow, most of which is occupied by leukemic cells Hematoxylin and eosin. × 1,500.

FIGURES 6 and 7.—Dividing leukemic cells from a peripheral blood smear. Giemsa × 1,500.





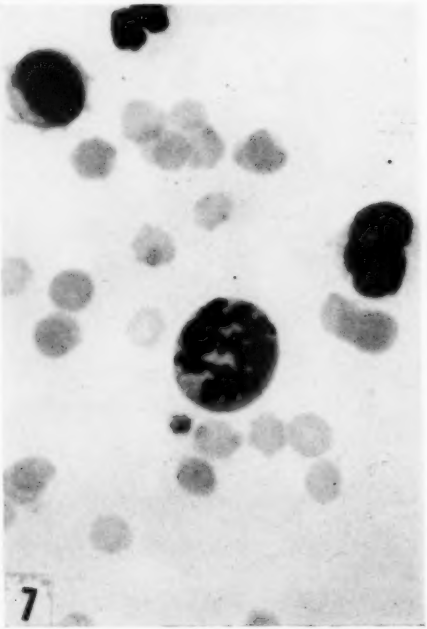


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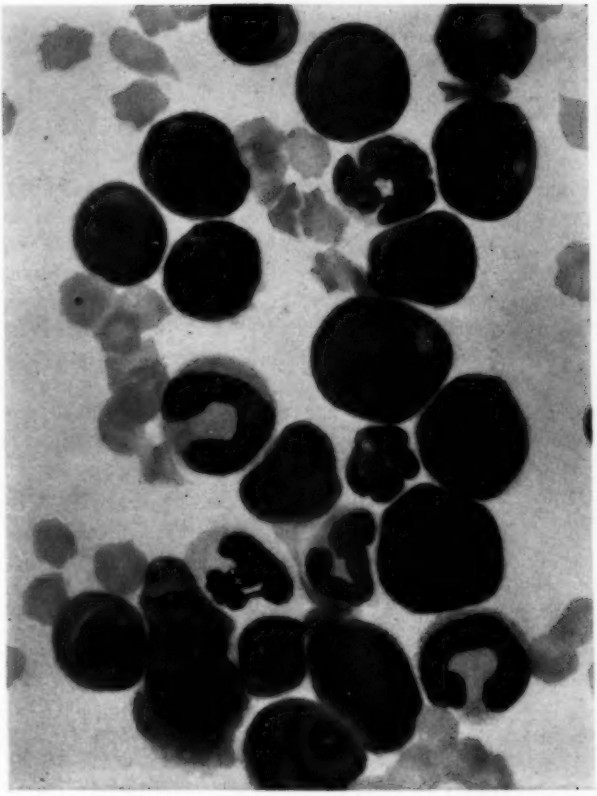
PLATE 66

FIGURE 8.—Peripheral blood smear. Large primitive cells (myeloblasts) comprised nearly one-half of the differential count. Immature intermediate granulocytic forms present in increased numbers. Giemsa. × 1,500.



Eyestone

HISTOLOGICAL EFFECTS OF LONG-CONTINUED WHOLE-BODY GAMMA-IRRADIATION OF MICE [1] [2]

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Long-continued exposure of laboratory animals to gamma radiation over the entire life span leads to injuries, many of them irreversible. The ultimate shortening of life depends upon the daily dose rate to which the animals are exposed. Under certain experimental conditions the predominant injury may consist of a lethal pancytopenia in one species and in other species of lethal tumor induction. In addition, in all species "premature aging" may result [Lorenz *et al.* (*1*)]. These investigators exposed several species of mammals daily to small doses of external gamma rays. The experiments were started at the beginning of maturity and continued for the remainder of the life span of the animals, which were killed only when moribund. This terminal condition was usually preceded by the sudden onset of progressive loss of weight or tumor formation, which was grossly evident. The data so obtained gave only terminal conditions.

To study progressive stages in the development of such changes, the experiments reported here were carried out; in them mice were exposed daily to one of three dose rates and then were sacrificed at intervals of 2 months.

MATERIAL AND METHODS

The mice used were of the same hybrid stock as used by Lorenz and co-workers (*1*). They came from the stock of the National Cancer Institute and were LAF_1 hybrids, obtained by crossing strain L females with

[1] Received for publication July 20, 1951.

[2] The work on which this report is based was started during the war by the Histological Group of the Metallurgical Laboratory (Plutonium Project) of the University of Chicago. The animals were irradiated at the National Cancer Institute by Dr. Egon Lorenz. He was also responsible for the repeated examination of the peripheral blood of the mice. The autopsies were done by members of the Histological Group who made periodic visits to Bethesda. The tissues were sectioned by technicians at the Argonne National Laboratory and turned over to us for study.

The study of this material was carried out under the supervision of Dr. William Bloom. We wish to thank Drs. Egon Lorenz and Leon Jacobson for their help in correlating some of our findings and for contributing their graphs showing the blood changes in these mice. We are also indebted to Drs. Eleanor Humphreys and Matthew Block for checking our conclusions on tumor and leukemia.

Part of the expense of this study was borne by a grant from the Dr. Wallace C. and Clara A. Abbott Fund of the University of Chicago.

strain A males. Hybrids were chosen because they are more vigorous and have a longer life span than inbred mice. Their spontaneous mammary tumor and other tumor incidence is low, with the exception of leukemia and lung tumors. Leukemia, however, is not observed until the animals are approximately 1½ years old. The incidence of leukemia for the entire life span is approximately 50 percent in females and 15 percent in males. Lung-tumor incidence is approximately 50 percent at the age of 18 months [Lorenz *et al.* (*1*)].

Exposure of the experimental animals was begun at the age of 2 months. Thirty-two male mice and 32 female mice were exposed at each of 3 dose levels: 1.1, 4.4, and 8.8 r daily (8 hours). A corresponding number of mice served as nonirradiated controls. Four male and 4 female mice of each dose level and the same number of controls were killed and autopsied at intervals of approximately 2 months up to 16 months. A few moribund animals were also studied. The mice were kept in wooden boxes, 8 animals in each. The boxes were made of pine board, 1 cm. thick, with inside dimensions of 28 x 18 x 15 cm. A small food hopper of galvanized iron was placed in the middle of each side board. The top of the boxes consisted of wire netting of 8-mm. mesh, nailed to a wooden frame. Drinking water was available to the mice in wide-mouth bottles of 100 cc. capacity, closed by a rubber stopper, into which a glass tube approximately 8 mm. in diameter and 10 cm. long was inserted. The bottles were supported by wire netting, with the drinking tubes approximately 5 cm. distant from the food hoppers along the midline of the cage. This arrangement was chosen to compel the mice to spend as much time as possible along the midline of the cage. The bedding of all animals consisted of sawdust, which was changed twice weekly; the cages were cleaned and sterilized once weekly. The mice were given Purina dog chow and water *ad libitum*.

The experimental arrangement for the irradiation of the mice has been described in detail by Lorenz (*1*). The source of radiation was radium sulfate contained in a capsule of platinum, 40 x 6.4 mm., 0.5 mm. thick. In equilibrium it has an external gamma radiation equivalent to that of 992.5 mg. of radium element. The gamma-radiation intensity at the midpoint of the boxes containing the mice was determined with Victoreen 0.25 and 25 r ionization chambers, and the boxes were so placed as to expose the mice to 8.8 r, or 4.4 r, or 1.1 r daily (8 hours). As the different points inside each box were variable distances from the source, the gamma-radiation intensity also varied from point to point, the maximum variations being about ± 10 percent. Since the mice were forced to feed and drink along the midline of the cages, the variations in total doses among the different experimental mice were probably somewhat smaller than ± 10 percent. The animals were irradiated for 8 hours daily, 7 times per week.

At the time of autopsy obvious deviations from normal, mainly tumors and lymphomas, were noted. The tissues fixed routinely did not include central nervous system, eye, mouth, thyroid gland, and hypophysis.

The tissues were fixed in Zenker-formol, embedded in nitrocellulose, sectioned, and stained with hematoxylin-eosin-azure II.

In the following pages, lymphoma is understood to include lymphosarcoma and all types of leukemia. We have often used the latter term when its connotation is clear.

OBSERVATIONS

Thymus Gland

Changes due to irradiation occurred at the three dosage levels and increased in intensity as the time and/or the dose of radiation was increased. In examination of irradiated and nonirradiated animals, several factors were checked to determine their usefulness in identifying the normal age changes. It was only after these were established, that the changes due to long-continued irradiation could be evaluated. The factors tested were: degree of atrophy of the thymus gland; the relative proportions of medulla to cortex, *i. e.*, the relative number of lymphocytes in the medulla and cortex; the relative increase in mast cells in the connective tissue around the gland, in the cortex, and in the medulla; the relative increase of plasma cells, green-staining pigment-containing macrophages; and mitoses and degenerating cells in the irradiated as compared to the nonirradiated animals. The sections were also checked for the presence of tumors.

Observations of the effects of irradiation of the thymus gland were restricted in some instances because of poor sectioning and because some of the sections through the thoracic viscera did not pass through the gland, especially in the late stages; possibly in some instances as a result of atrophy due to age or irradiation. The irradiation changes, mainly increase in mast cells and lymphocytic depletion, were not always completely consistent among the individual animals of a given group; but entire groups could be identified as irradiated or not, or as young or old controls. It was difficult to distinguish between animals irradiated at different dose rates unless the duration of treatment was also known.

Untreated control mice.—With increasing age beginning at 12 months the ratio of medullary to cortical areas was increased, signifying a depletion of lymphocytes in the cortex. At 16 months the one slide available showed no difference in the density of lymphocytes throughout the organ.

With increasing age the number of mast cells in the connective tissue surrounding the gland, in the cortex, and in the medulla increased, starting at 2 to 4 months. The increase was most striking in the cortex. As serial sections were not available, counts of large numbers of areas could not be made. Nevertheless, there was no doubt that an actual increase had occurred.

Plasma cells and green-staining pigment-containing macrophages appeared at 4 months. There was a slight increase in the number of green-staining pigment-containing macrophages in the older animals. No consistent change in the number of plasma cells was noticed after 4 months.

In many instances the degree of atrophy of the organ could not be estimated, owing to irregularities in sectioning. The numbers of mitotic and degenerating cells were not indicative of age changes. No tumors (including lymphomas) were found in any controls. No differences were

noted between the sexes with respect to onset or degree of age changes.

Findings in irradiated animals.—1.1 r daily (8 hours): In these animals the acceleration of the depletion of cortical lymphocytes over those of the controls was first noticed at 10 months. This depletion was more marked at 12 months. At 14 months, although there was definite depletion in the controls, the 1.1 r animals showed no depletion. At 16 months this difference was even more marked, since the control was extremely depleted. Proliferation of lymphocytes in the cortex at 14 and 16 months was taking place in the irradiated mice.

A definite increase in the number of mast cells occurred in the females at 10 months, with a probable increase in the males at the same time. The mast-cell count in the medulla was three or four times that of the controls. The increase persisted at 12 months, but fewer mast cells were present at 14 and 16 months.

4.4 r daily (8 hours): An acceleration of depletion of cortical lymphocytes was first noticed at 8 months. At 10 months this was more marked than in the 1.1 r animals. At 12 moths no depletion of lymphocytes was noticed, but at 14 months there was a slight decrease, and at 16 months the section could not be differentiated from those of the controls of the same age on this basis.

At 8 months there was a definite increase in mast cells, of the same order of magnitude as in the animals irradiated with 1.1 r, but it climbed steadily throughout the ensuing period of radiation to reach a maximum at 16 months.

8.8 r daily (8 hours).—Depletion of cortical lymphocytes greater than that in the controls was noted at 6 months. At 8 and 10 months the depletion was more marked than that of the 4.4 r group. At 12 months one specimen was very depleted, while the other two were leukemic.

At 14 months sections from the two mice showed that both were leukemic. At 16 months the thymus gland of one mouse was leukemic, while the other showed no decrease in lymphocytes as compared to the normal of the same interval. Presumably proliferation of lymphocytes had occurred.

The mast-cell count was increased over the controls at 4 to 6 months, and the increase persisted up to and including the nonleukemic animal at 12 months. In the two leukemic animals at 14 months mast cells were markedly decreased as well as in the 14-month animals at 4.4 r. At 16 months the mast-cell count was low in the leukemic animal and high in the nonleukemic animal.

Thus, leukemia was present in five animals irradiated with 8.8 r: two females at 12 months, two males at 14 months, and one female at 16 months. The leukemic changes were characterized by extreme hyperplasia of medium and large lymphocytes in the cortex and medulla. As the greater increase took place in the medulla, the normal medullocortical demarcation was abolished. There was a large increase in the number of mitotic and degenerating cells and a great reduction in the number of mast cells.

Summary.—In the thymus gland these doses of chronic gamma irradiation produced an increase in the number of mast cells and a decrease in the number of lymphocytes in both cortex and medulla. Both changes began at 10 months with 1.1 r daily (8 hours); at 8 months with 4.4 r daily (8 hours); and at 4 to 6 months with 8.8 r daily (8 hours). Lymphocytic regenerative attempts were present at 14 and 16 months in the animals exposed to 1.1 r and 4.4 r daily (8 hours). In the latter group the destructive and regenerative tendencies were more closely balanced. Histologically, lymphocytic regeneration seemed to merge into lymphoma in some of the older animals receiving 8.8 r daily (8 hours). Sex differences were not observed.

Spleen

The spleen was one of the more responsive organs in this range of radiation. Variations from animal to animal were marked, so that study of a whole group was necessary to get a representative picture. In some cases there was so much variation that it was difficult to be positive whether extreme hyperplastic changes were regenerative or preleukemic in nature. In addition, in some groups of eight animals receiving the same amount of radiation over the same period, one or two responded markedly, whereas one was indistinguishable from control animals, and the others were intermediate.

The most striking change in the spleen of the mice receiving 4.4 r and 8.8 r daily (8 hours) was the great increase in the number of mast cells in contrast to the controls, which showed no change with age. Although there were some individual variations in the number of mast cells in the spleens, the numbers of these cells in the spleens of each group as a whole showed little variation. In the animals receiving 4.4 r daily (8 hours) there was a regular increase beginning at 8 months and progressing to 16 months, when a maximum of three times the numbers in the controls was reached. Mice receiving 8.8 r daily (8 hours) showed an increase from 4 to 16 months, with a maximum of 4½ times the control.

The ratio of red to white pulp was measured by making camera lucida drawings of the outlines—in the sections—of the spleen and white pulp upon bond paper. The outline of the spleen was trimmed and weighed on an analytical balance. The areas representing white pulp were cut out and the remainder weighed. Subtraction of the latter weight from the former gave a figure for the white pulp. By comparing the weight representing the total spleen with the weight representing the white pulp, the percentage of total area made up by the white pulp was obtained.

The untreated mice showed a gradual, progressive increase in the percentage of white pulp, reaching 26 percent at 8 months, 28 percent at 10 months, 29 percent at 12 months, and 30 percent at 16 months. The animals treated with 8.8 r daily (8 hours) showed a progressive increase in the percentage of white pulp from 30 percent at 8 months to 32 percent at 10 months, 38 percent at 12 months, and 42 percent at 16 months. Animals treated with 4.4 r showed 31 percent at 14 months and 34 percent

at 16 months, a slight increase over controls. The increases are statistically valid, except for the 8-month stage after 8.8 r and the 14-month after 4.4 r, which are borderline. However, in the over-all picture these changes seem significant. There was no obvious change in the percentage of white pulp in the 1.1 r per day mice.

Accompanying the increase in white pulp there was a gradual trend toward its becoming less dense. This was accompanied by a relative increase in medium lymphocytes, reticular cells, large lymphocytes, and plasma cells. In the 14- and 16-month animals exposed to 8.8 r typical nodules no longer appeared, although they were prominent at 12 months. The most responsive animal receiving 8.8 r for 16 months showed a white pulp consisting predominantly of medium lymphocytes with reticular and plasma cells. The borders were not the well-limited type found ordinarily but extended irregularly into the red pulp.

The controls showed no marked changes, except an increase in nodules with age.

There was an increase in erythropoiesis of irradiated animals compared with controls. Animals exposed to 4.4 r showed an increase at 14 and 16 months. Those exposed to 8.8 r showed an increase at 6 months, which became progressively greater up to 16 months. The 16-month animals exposed to 8.8 r showed large areas of red pulp populated entirely by hemocytoblasts, compared to occasional groups of two or three found in controls of this age.

The red pulp showed an increase in granulocytes in the 14- and 16-month group exposed to 8.8 r, though this was not nearly as marked as the change in the red-cell series.

Measurements of the sections with a millimeter rule showed an increase in size at 12, 14, and 16 months for 1.1 r, 4.4 r and 8.8 r, but no increase in the controls. Because of irregularities of the sections and the lack of serial sections this measurement, although progressively greater with higher doses, may not be entirely reliable.

Summary.—Changes in the spleen due to irradiation were noted only at the two higher dose levels. There was an increase in the number of mast cells in the 4.4 r series beginning at 8 months and reaching a maximum of three times the number in controls at 16 months, in the 8.8 r series beginning at 4 months and reaching four and one-half times the number in controls at 16 months. With increasing length of exposure time, the white pulp occupied a progressively larger percentage of the spleen as measured in sections, at the same time becoming less dense, due mainly to its depletion of small lymphocytes. It was characterized by an increase in the relative number of medium-sized and large lymphocytes, reticular cells, and plasma cells, and the absence of nodules at late intervals. In the red pulp increased erythropoiesis began in the 4.4 r series at 14 months, in the 8.8 r series at 6 months, progressing to 16 months.

Bone and Bone Marrow

The tibia was examined for bone and for marrow. The average length of this bone in mice of a treated group was found to be comparable to

that of the similar age control group. There were no microscopic changes in either the cellular or intercellular components of the bone as a result of irradiation.

To determine whether there were any changes in the marrow that might have been due to irradiation, counts (tables 1 and 2) were made using the Chalkley method (*2*). Five pointers were placed in the eyepiece of the microscope. One pointer was used to focus and locate new fields, and the spaces or cell types at the other four pointers were recorded for each position. The slide was moved in a random manner along the length of the bone marrow until 100 fields were counted for each animal. The cells counted were classified as in tables 1 and 2. The first column listed as "space" included fat cells, blood vessels, and noncellular areas. Animals with lymphoma listed in table 4 were omitted from these counts.

The most marked change was the increase in the number of mast cells, which, as can be seen in the accompanying tables, were practically absent from the control animals. The greatest increase was in the middle of the shaft. The trend shown in table 3 is in agreement with separate counts made in the area of highest concentration. Sections from animals subjected to 4.4 r for 10 months showed an increase that was progressively greater with increased time of exposure, the maximum being 10 per high-power field at 16 months. Sections from animals receiving 8.8 r showed an increase in mast cells beginning at 6 months regularly to a maximum of 36 per high-power field at 16 months. These increases parallel those seen in the spleen but begin 2 months later and do not rise as rapidly or as much.

There was no change in the megakaryocyte population. The ratio of the areas occupied by the red- and white-cell series of the bone marrow did not change in irradiated animals. The number of hemocytoblasts was definitely increased in animals receiving 8.8 r for 8 months and 12 months (see table 3). Degenerating cells and pigment-containing macrophages were not more numerous than in the control animals.

There was an increased gelatinous replacement of the cellular elements of the marrow in a diffuse long pattern beginning at the metaphysis and extending various distances down the shaft. This condition was found very rarely in a control animal but in treated animals receiving higher doses it was a constant finding. Animals receiving 4.4 r show this beginning at 6 months and animals receiving 8.8 r beginning at 4 months.

The leukemic changes involving the marrow are summarized in the section on tumors and leukemia.

To summarize, the changes in the bone marrow include a gelatinous replacement of the cellular marrow, especially in the metaphysis, a progressive increase in the number of mast cells and, at the highest dose level, an increase in the number of hemocytoblasts. The extent of these changes was greater with 8.8 r than with 4.4 r and with duration of treatment. No changes were seen after 1.1 r daily (8 hours).

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TABLE 1.—*Cellular constitution of bone marrow of control LAF$_1$ mice*

Interval	Animal No.	Space	Red-cell series	White-cell series	Reticular	Macrophages	Megakaryocytes	Hemocytoblasts	Mast	Plasma	Mitosis
4 months	12657	112	109	128	9	11	13	16	0	2	0
	12658	107	88	152	8	9	16	15	0	4	1
	12659	105	131	116	5	12	17	9	0	4	1
	12660	115	128	120	5	6	14	10	0	2	0
	12661	85	145	132	4	5	9	16	0	3	1
		105	120	130	6	9	14	13	0	3	1
8 months	12709	92	84	160	12	8	22	15	0	6	1
	12710	95	96	148	11	20	11	13	0	5	1
	12711	98	102	134	10	14	20	18	0	4	0
	12712	108	130	114	4	18	6	16	0	3	1
	12705	117	86	138	8	11	16	20	0	4	0
	12706	112	105	123	8	20	14	12	1	5	0
	12708	88	130	126	7	15	12	18	0	3	1
		101	105	135	9	15	14	16	0	4	1
12 months	12776	64	105	154	18	21	15	14	0	7	2
	12777	80	103	146	20	17	15	14	0	5	0
	12779	99	111	118	18	20	14	14	0	5	1
	12780	80	79	173	17	21	13	10	1	6	0
		81	100	148	18	20	14	13	0	6	1
16 months	12838	148	111	113	15	1	6	5	0	1	0
	12840	140	110	118	9	5	8	4	0	6	0
	12841	87	89	175	17	8	14	7	0	3	0
	12843	85	129	151	12	3	7	8	0	3	2
	12844	129	120	112	7	11	12	8	0	1	0
		118	112	134	12	6	9	6	0	3	0

TABLE 2.—*Cellular constitution of bone marrow of LAF$_1$ mice receiving 8.8 r daily (8 hours)*

Interval	Animal No.	Space	Red-cell series	White-cell series	Reticular	Macrophages	Megakaryocytes	Hemocytoblasts	Mast	Plasma	Mitosis
4 mo. (8.8 r)	12649	85	122	146	5	7	17	16	1	1	0
	12650	103	137	115	9	3	13	14	2	3	1
	12652	138	112	109	7	3	17	10	0	4	0
	12653	99	129	144	2	2	13	9	0	2	0
	12654	91	146	125	4	7	7	17	0	3	0
	12655	113	128	113	9	4	12	15	0	6	0
	12656	88	112	160	8	13	9	9	0	1	0
		106	125	130	6	6	12	12	0	3	0
8 mo. (8.8 r)	24221	98	116	118	5	17	23	11	6	4	2
	24222	115	114	111	6	21	17	10	2	3	1
	12757	100	93	138	7	12	15	28	1	4	2
	12758	115	95	132	4	7	17	24	0	5	1
	12759	142	98	106	8	11	11	19	0	4	1
	12760	87	129	107	12	6	18	35	0	5	1
		109	108	119	7	12	17	21	1	5	1
12 mo. (8.8 r)		78	94	151	12	20	7	22	5	6	5
	12803	70	103	128	21	21	12	30	8	3	4
	12804	103	115	90	10	25	15	28	8	3	3
	12805	136	71	129	11	16	5	12	14	3	3
	12800	106	103	90	14	22	18	37	2	6	2
		99	97	118	14	21	11	26	7	4	3
16 mo. (8.8 r)	24211	140	71	131	8	5	14	12	18	1	0
	24212	78	97	102	40	6	18	26	10	17	6
	24213	114	83	135	13	8	10	10	15	11	1
	24215	125	109	134	9	5	11	5	2	0	0
		114	90	128	17	6	13	13	11	7	2

TABLE 3.—*Summary of tables 1 and 2*

CONTROLS

Interval	Space	Red-cell series	White-cell series	Reticular	Macrophages	Megakaryocytes	Hemocytoblasts	Mast	Plasma	Mitosis
Totals:										
4 months	105	120	130	6	9	14	13	0	3	1
8 months	101	105	135	9	15	14	16	0	4	1
12 months	81	100	148	18	20	14	13	0	6	1
16 months	118	112	134	12	6	9	6	0	3	0
TREATED										
Totals:										
4 months	106	125	130	6	6	12	12	0	3	0
8 months	109	108	119	7	12	17	21	1	5	1
12 months	99	97	117	14	21	11	26	7	4	3
16 months	114	90	128	17	6	13	13	11	7	2

MESENTERIC LYMPH NODE

The mesenteric lymph node in animals irradiated at 8.8 r daily (8 hours) showed progressive changes beginning in the 10-month group and extending through the 16-month group. However, there were no changes to be observed in the animals subjected to this amount of irradiation for 2 to 10 months. Those irradiated at 1.1 r and 4.4 r daily (8 hours) showed no changes different from those in the control group.

Starting at 10 months and extending through the 16-month group irradiated at 8.8 r daily (8 hours) there was a gradual decrease in the number as well as the size of the lymphocytopoietic centers, which disappeared completely at 16 months. There was also a progressive decrease in the number of cortical sinuses packed with small lymphocytes until there were none left at 16 months. The number of small lymphocytes in the medullary sinuses decreased slightly.

Other changes of lesser degree occurred at the same interval and dose rate [10 to 16 months, 8.8 r daily (8 hours)]. Among these was an increase in dense areas of reticular cells in the cortex. Although such areas were also seen in the controls, the increase was much greater in the irradiated animals. A few macrophages contained a green-stained pigment (predominantly in the medulla). Such cells were not observed in the controls or in groups of animals irradiated to a lesser degree. There was a diffuse depletion of lymphocytes, predominantly in the internodular portion of the cortex.

Cell counts were not made, since the random plane of sectioning of these small organs prohibited this type of analysis. Indeed, even with an ideal plane of the sectioning, the inconstant morphologic appearance of this tissue would tend to invalidate the accuracy of such counts.

Of the many characteristics of the mesenteric lymph node, the ones associated with lymphocytopoietic activity and transport of small lymphocytes tended to show the greatest changes following irradiation. However, some characteristics that may or may not be associated with this phenomenon should be mentioned here. Since mast cells were found mostly in the medulla and many of the sections consisted mainly of cortex, an increase in mast cells could not be demonstrated, in contrast to the findings in thymus gland, bone marrow, and spleen. Also, many of the sections of lymph node showed no mast cells in the medulla; accordingly, no conclusion could be drawn with regard to this characteristic.

The number of eosinophils remained fairly constant in both control and irradiated animals, with the exception of a few mice with parasitic infection, in which they showed a vast increase in localized areas. Plasma cells showed no change that could be correlated with increased doses of irradiation or that could not be demonstrated in the control animals of the same age.

The number of small lymphocytes in the medullary cords tended to show a slight decrease in the mice irradiated for 10 to 16 months with 8.8 r daily (8 hours), but the change was not striking.

The subcapsular sinus varied in size from animal to animal (control and irradiated), and invasion of the sinus by small lymphocytes appeared in most animals.

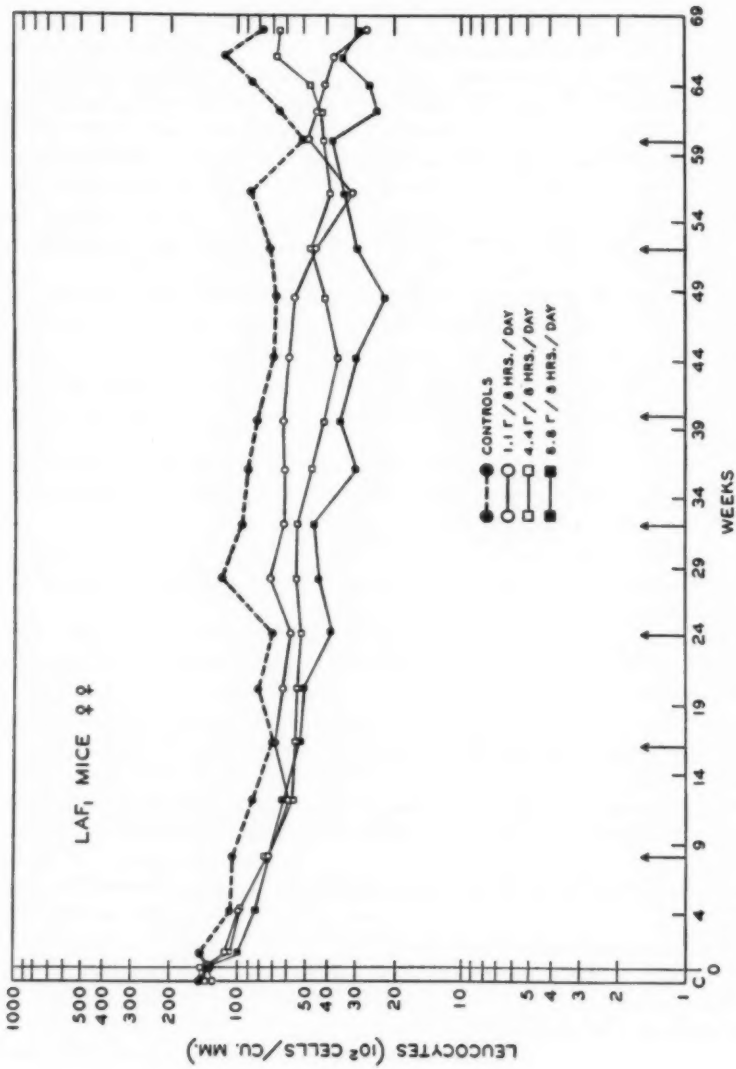
The lymph nodes showed no variation with the sex of the irradiated or control animals, with the exception of a leukemic tendency which was found mostly in the female.

Age changes of the controls did not simulate any of the major changes due to irradiation. Although there did not appear to be as many small lymphocytes in the cortex of the older control animals, as compared to the younger ones, lymphocytopoietic centers, as well as cortical sinuses packed with small lymphocytes, could be demonstrated in all of the controls.

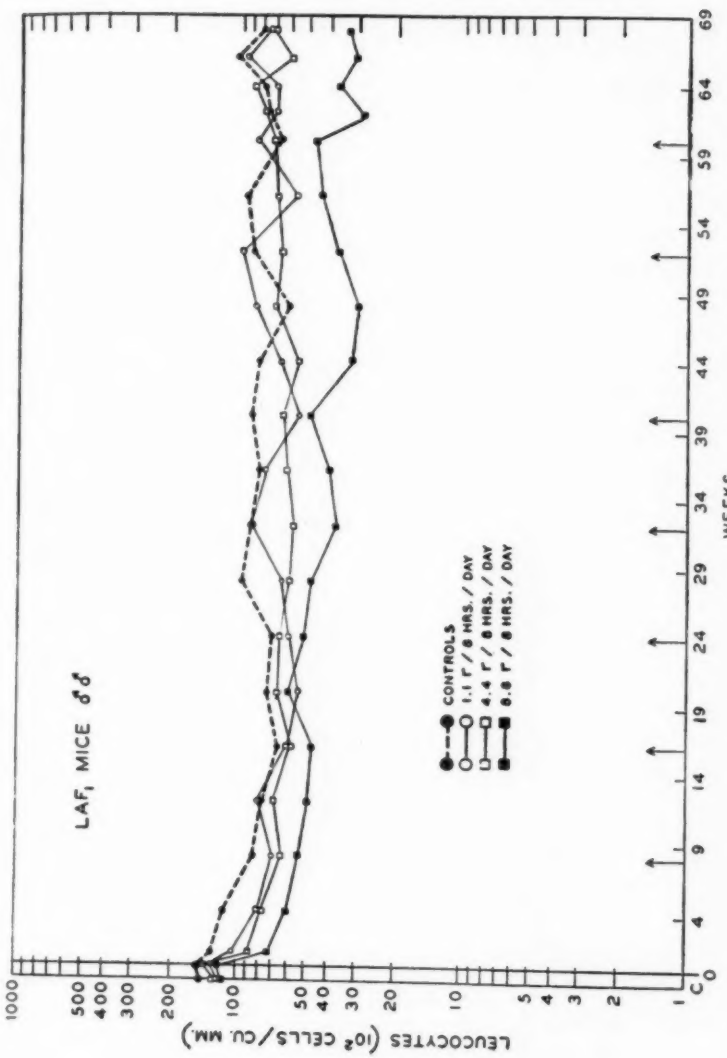
It is significant that the major irradiation changes observed in the lymph node, *i. e.*, decrease in lymphocytopoiesis and in the number of small lymphocytes packed in the cortical sinuses, correlate remarkably with the decrease in lymphocytes in the peripheral blood counts of the animals irradiated at 8.8 r daily (8 hours) for 10 to 16 months. The decrease in the peripheral lymphocyte count in animals irradiated at 8.8 r daily for 2 to 10 months was gradual and was not reflected in changes in the lymph nodes (text-figs. 1–12).

In the period of greatest change, 10 to 16 months, 8.8 r daily (8 hours), evidences of leukemia were seen in four mesenteric lymph nodes, one each at 10, 12, 14, and 16 months, all in females.

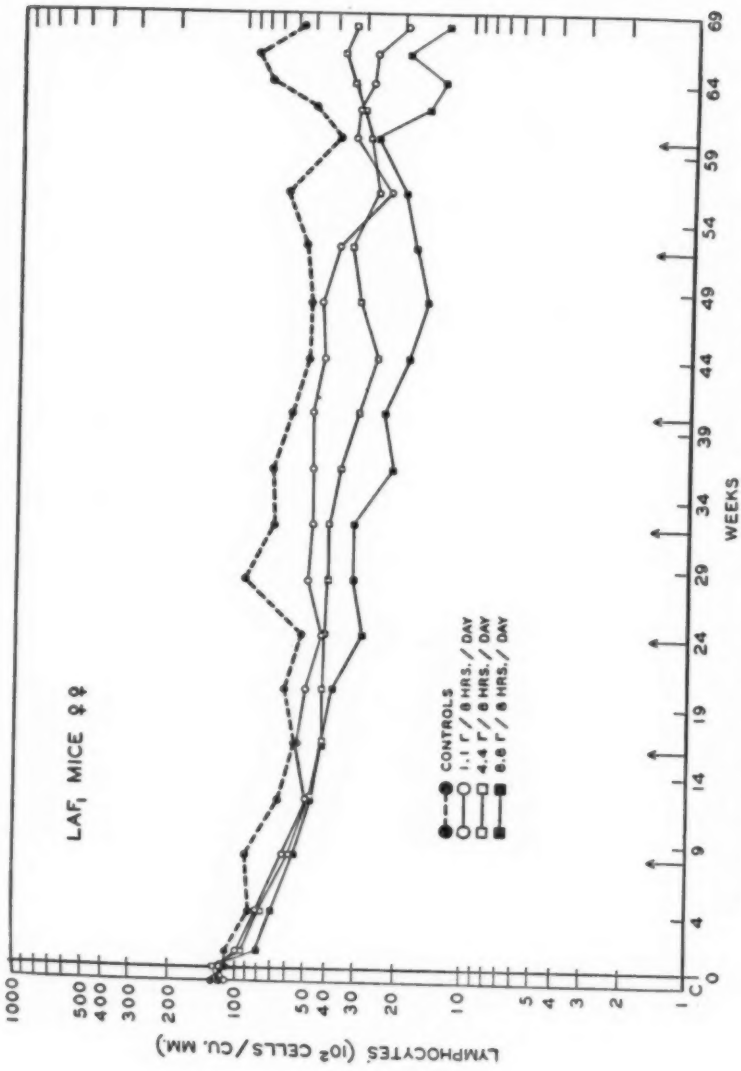
Summary.—Changes were observed only in the animals irradiated with 8.8 r daily for 10 to 16 months. In the lymph nodes of these mice there was a decrease in the number of small lymphocytes in both cortex and medulla and in the number and size of lymphocytopoietic centers as well as of cortical sinuses packed with small lymphocytes.



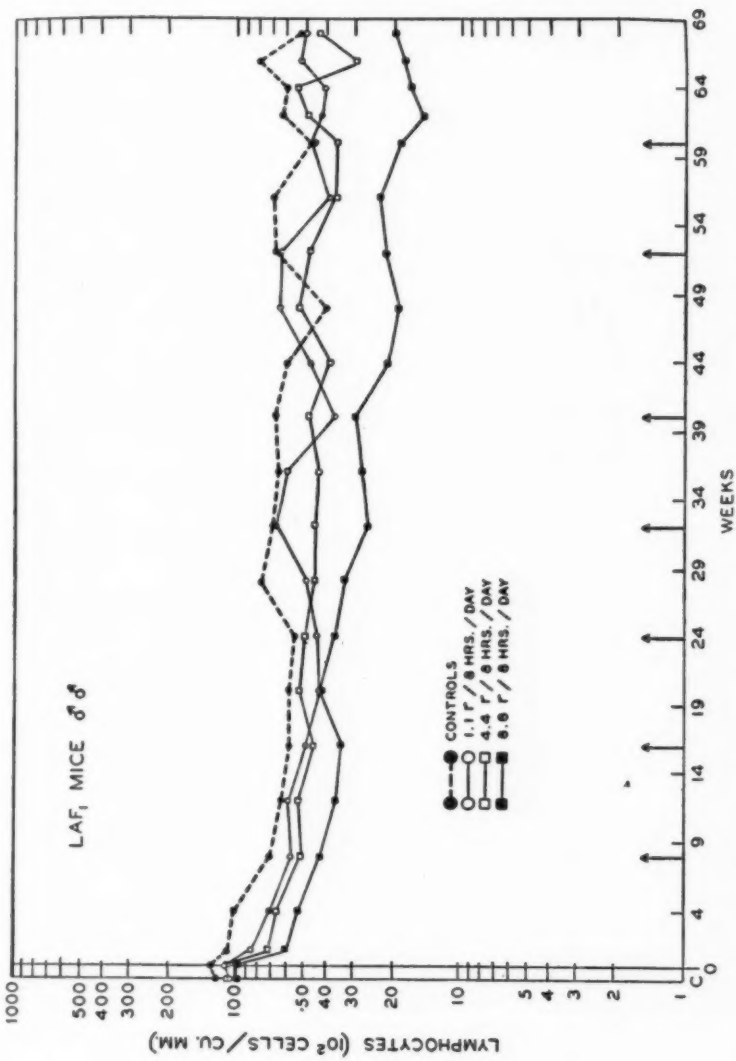
TEXT-FIGURE 1.—Leukocyte counts of peripheral blood of LAF_1 female mice.



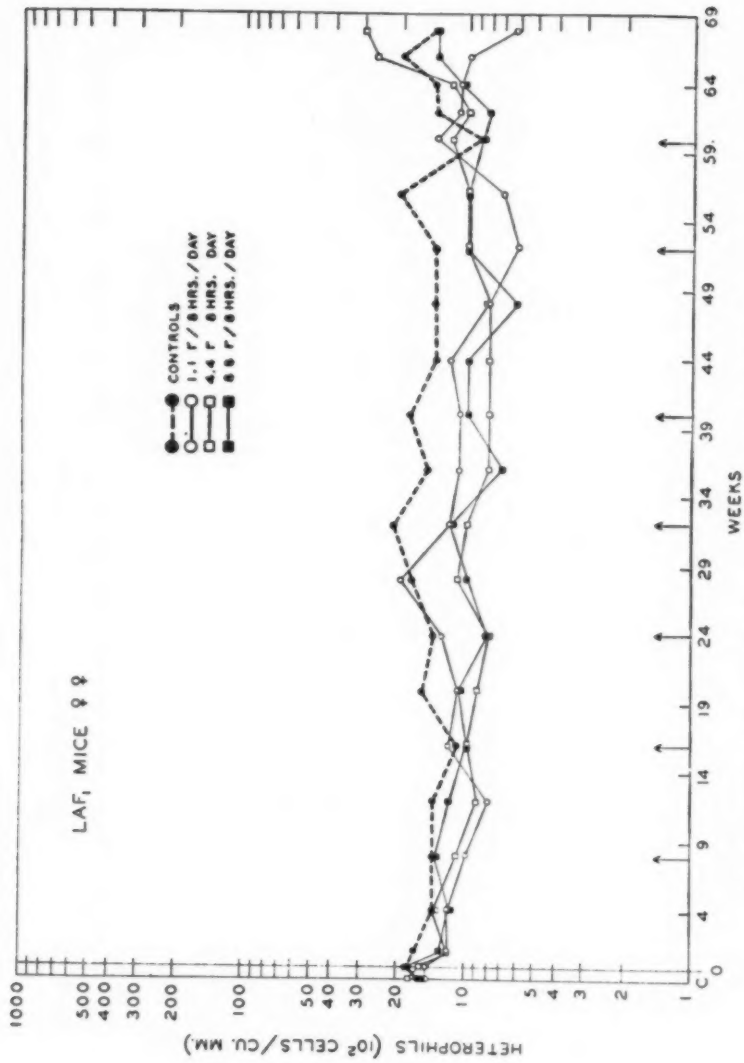
TEXT-FIGURE 2.—Leukocyte counts of peripheral blood of LAF_1 male mice.



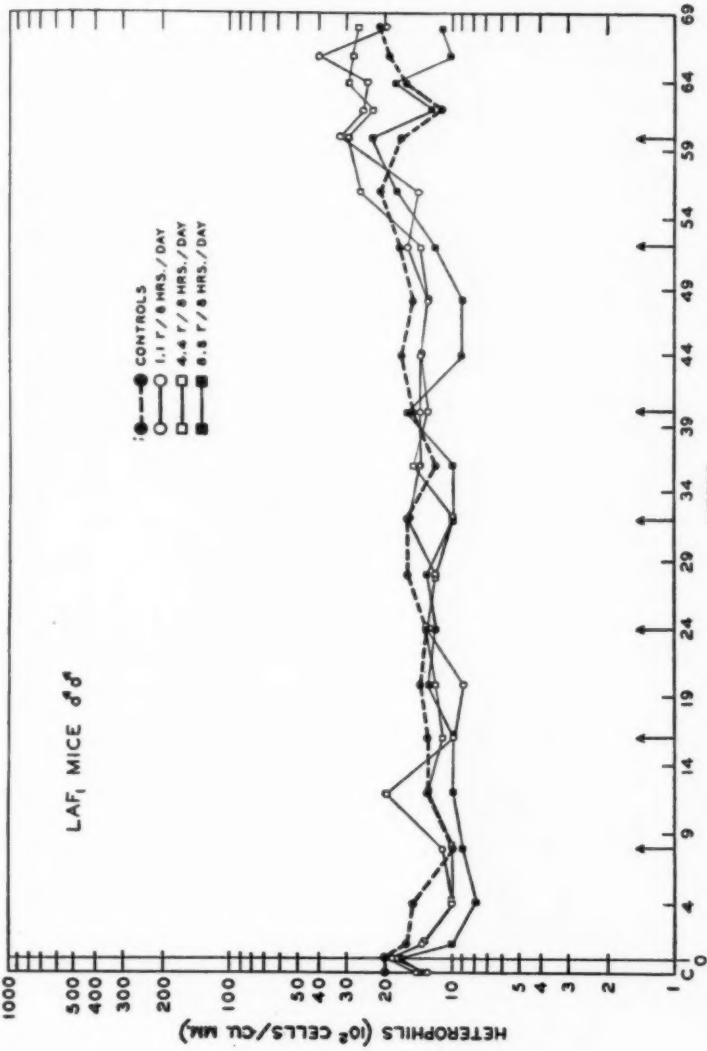
TEXT-FIGURE 3.—Lymphocyte counts of peripheral blood of LAF_1 female mice.



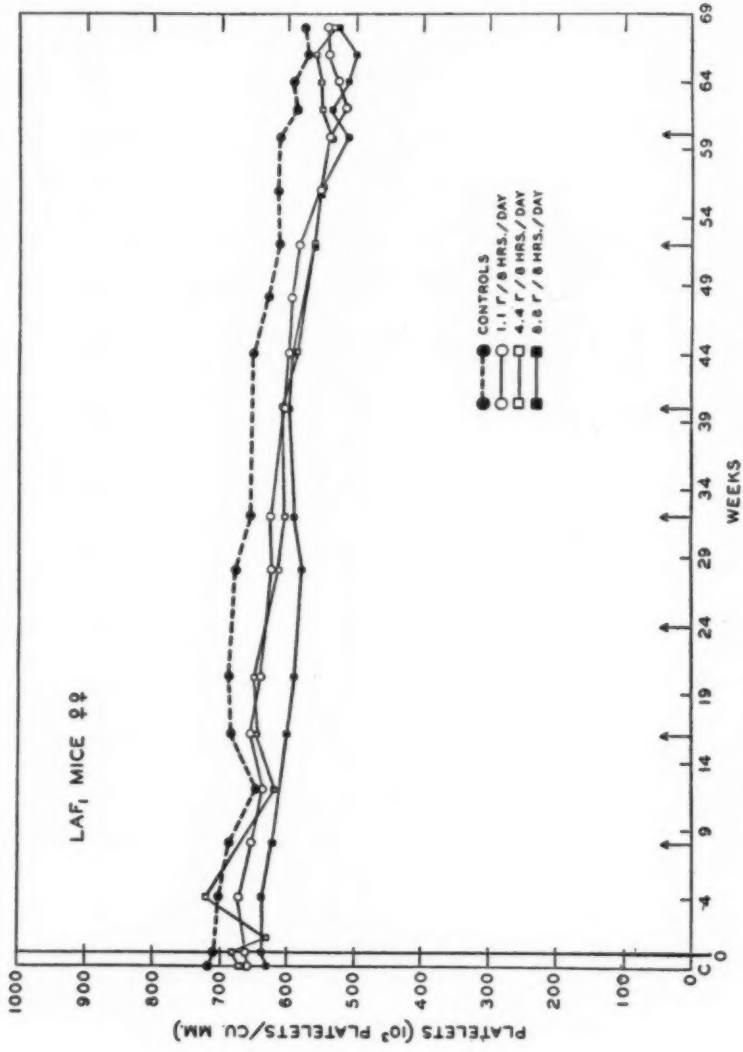
TEXT-FIGURE 4.—Lymphocyte counts of peripheral blood of LAF_1 male mice.



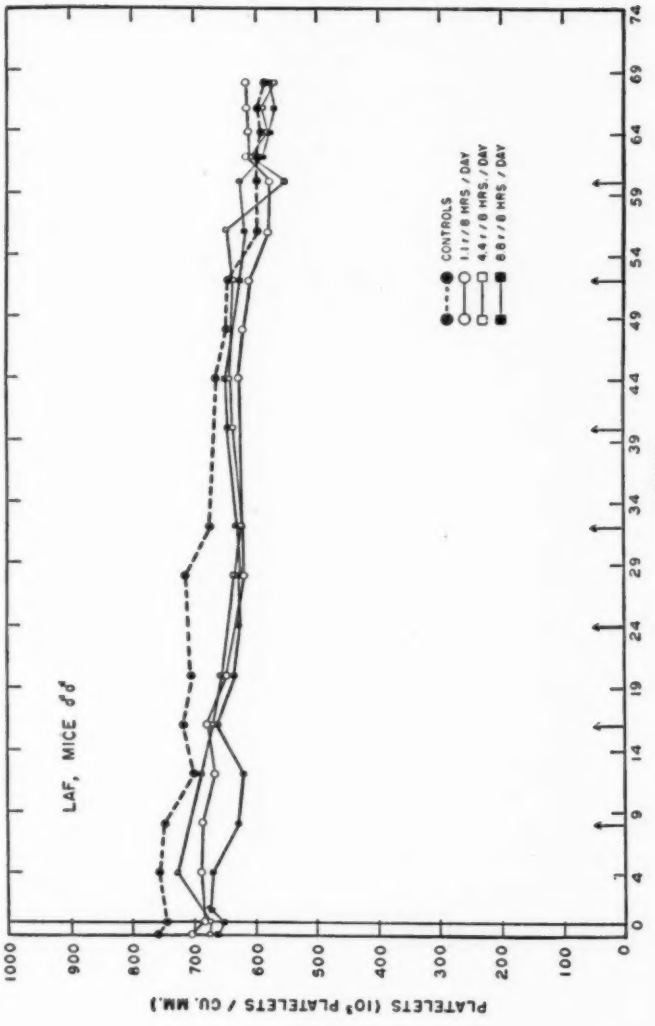
TEXT-FIGURE 5.—Heterophil counts of peripheral blood of LAF_1 female mice.



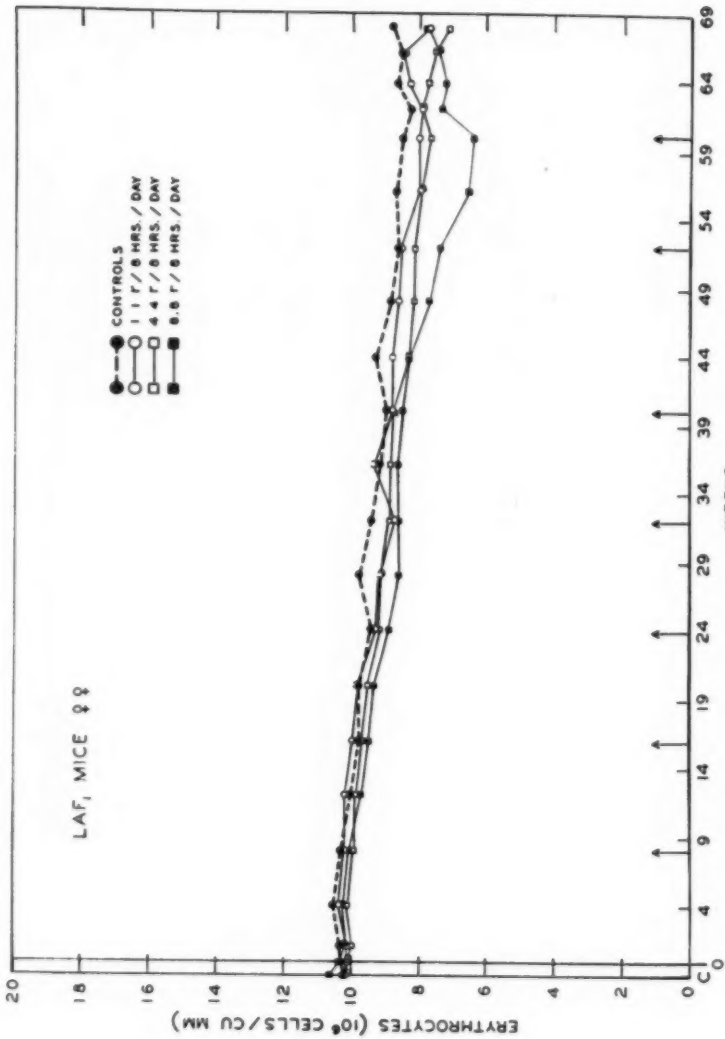
TEXT-FIGURE 6.—Heterophil counts of peripheral blood of LAF_1 male mice.



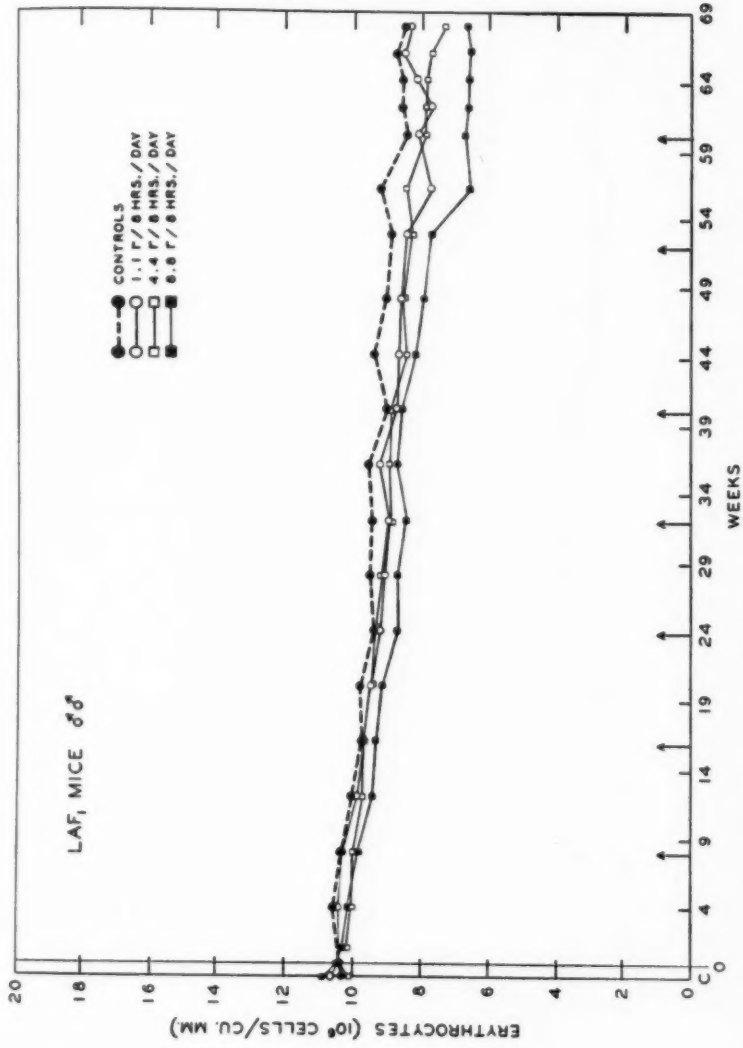
TEXT-FIGURE 7.—Platelet counts of peripheral blood of LAF_1 female mice.



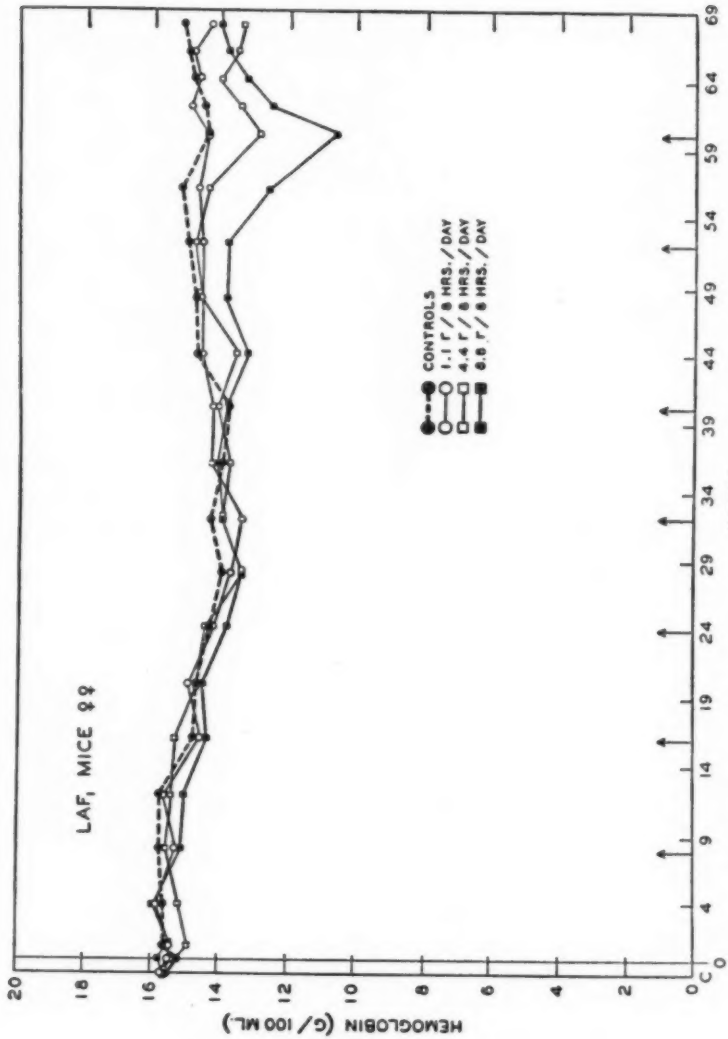
TEXT-FIGURE 8.—Platelet counts of peripheral blood of LAF_1 male mice.



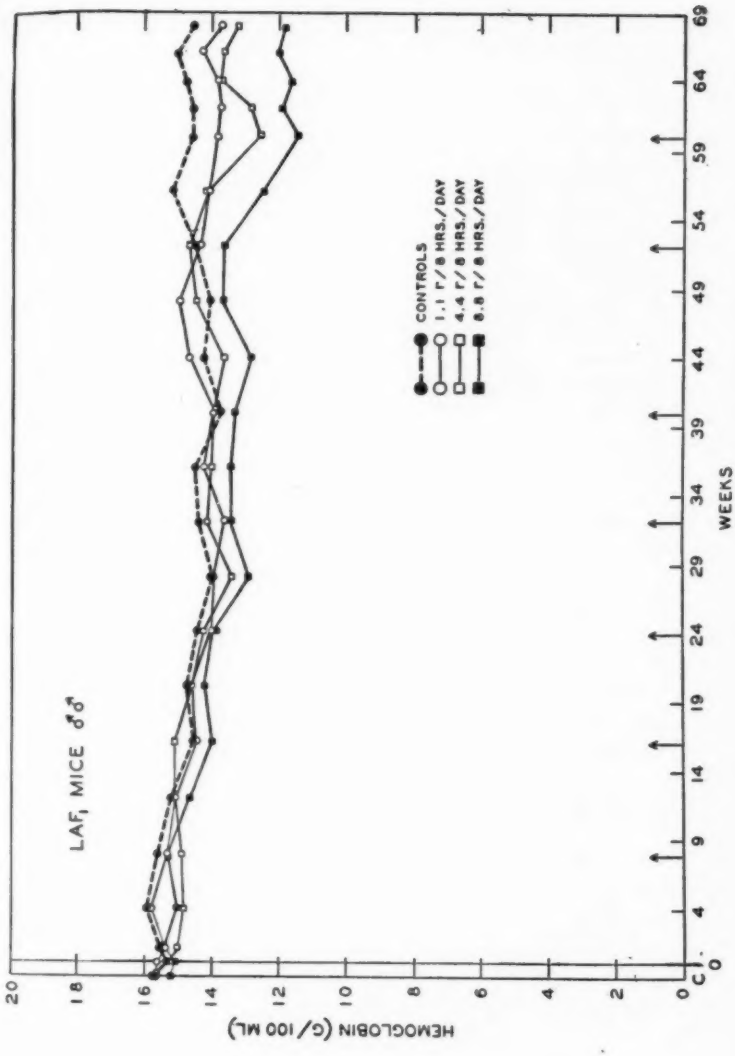
TEXT-FIGURE 9.—Erythrocyte counts of peripheral blood of LAF$_1$ female mice.



TEXT-FIGURE 10.—Erythrocyte counts of peripheral blood of LAF_1 male mice.



TEXT-FIGURE 11.—Hemoglobin determinations of peripheral blood of LAF_1 female mice.



TEXT-FIGURE 12.—Hemoglobin determinations of peripheral blood of LAF_1 male mice.

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The marked decrease in lymphocytes paralleled the lymphocytopenia in the peripheral blood at this time.

Findings in the Peripheral Blood

Counts made on the peripheral blood of these animals were analyzed by Drs. Egon Lorenz and Leon Jacobson, and have been reported by them in the Argonne National Laboratory Quarterly Report, May–July 1949 (*3*). With their kind cooperation, and for the sake of completeness, these charts are reproduced here. This seems desirable since the Argonne National Laboratory Quarterly Reports are not widely circulated.

Blood data were obtained on all animals. Counts were made 1 week prior to exposure, the day the exposure was started, at the end of the first week of exposure, and every 4 weeks thereafter. In addition, weight records were kept of all animals. These data are presented here because more frequent counts were taken in the early part of this experiment than in the original experiment of Lorenz *et al.* (*1*) on guinea pigs, rabbits, rats, and mice. Furthermore, more animals were available during the first 6 months in the present series. The data (text figs. 1–10) confirm the findings of the original experiments of Lorenz *et al.* that the total leukocyte and lymphocyte counts are significantly depressed only in the 8.8 r groups of both males and females, and that this depression is established early and maintained throughout the experiment. The lymphocytes are depressed in the males receiving 4.4 r daily below those in the controls. It is not clear whether this depression is significant. The heterophil-leukocyte and platelet counts are probably not significantly lowered in any group of either males or females. The red-cell counts and hemoglobin values are reduced only in the animals exposed to 8.8 r daily for about a year. The counts on animals that developed leukemia are not included in these graphs.

The Testis

Eschenbrenner, Miller, and Lorenz (*4*) have reported the findings of a quantitative histologic analysis of the right testes of the mice used in this experiment. The present report is of the histologic observations made on the left testes of the same mice. Although this work consisted for the most part of subjective microscopic observations, some crude measurements were made. The capsule thickness was measured with a micrometer ocular. A rough estimate of the area of the tissue sections was made by measuring with a millimeter rule the 2 axes of the elliptical sections of the testes. The incidence of the presence of globules in the nucleus of the Sertoli cells was estimated by counting 50 cells in each section, not more than 5 being counted in each high-power field.

In general, the spermatogenic elements of the mouse testis resemble those of testes from other mammalian species. The prespermatogonial cells found in the mouse are located close to the basement membrane and may be classified and described as follows:

Stem cells.—These cells (figs. 1, *B* and *C*) are not seen in every section

of a tubule but as many as six may be seen in some tubules. They possess ovoid nuclei roughly the size of those of Sertoli cells. The karyoplasm is light-staining and vesicular, and there are two to six accumulations of chromatin material within the nucleus. The basophilic cytoplasm is scanty along the sides of the ovoid nucleus and extends into long tapering points (fig. 1).

Prespermatogonia.—In this category there is a variety of cell shapes in a series ranging from thin and fusiform to the globular, obviously resting spermatogonia. All cells are small, being roughly the size of the dividing spermatogonia. The nucleus may at times be darker than the basophilic cytoplasm in the globular cells, lighter in the fusiform ones, and there are up to a dozen very dark dots throughout the nucleoplasm. These cells (fig. 1*A*) are almost as plentiful as the dividing spermatogonia.

Changes in seminiferous epithelium.—1.1 r daily (8 hours) series:—Testes from mice throughout this series did not differ from the testes of non-irradiated mice.

4.4 r daily (8 hours) series:—For the most part testes from this group appeared normal, except for a few singly located, degenerate tubules that were seen in each section at all stages, there being slightly more damage after 12 months of irradiation. These tubules typically exhibited a loss of spermatogenic elements, with resulting prominence of the Sertoli syncytium, and degenerate pyknotic Sertoli-cell nuclei. In the 2- to 10-month groups about 3 such degenerate tubules per section were found. In the 12- to 16-month groups there were progressive, slight increases in degeneration. This was manifested by the appearance of vacuoles in the Sertoli syncytium of the degenerate tubules and by an increase in the number of such tubules per section. About 10 tubules per section were degenerate in the 16-month group. There was also a generalized, slight decrease in the height of the tubular epithelium and a slight, relative increase in the ratio of the interstitial tissue to the tubules.

8.8 r daily (8 hours) series:—Testes of the mice from this group showed considerable inhibition of spermatogenesis even after 2 months' irradiation. This progressed in the succeeding age groups and in the 8- to 16-month groups almost all tubules were practically devoid of cellular elements other than Sertoli cells, some of which were shrunken.

An exposure for 2 months at this rate resulted in a greater degree of change than that observed after 16 months of irradiation at the rate of 4.4 r daily. However, no single tubule was damaged to the same degree as were some at all stages of the 4.4 r series. The effect, then, was a generalized one although the tubules did show a wide range in degree of damage. On the whole, the tubules appeared smaller than those in the untreated testes and although they looked relatively well packed, there was a decrease in the number of spermatogenic cells.

From the 2-month group to the 8-month group there was a progressive increase in damage, so that the 8-month group presented a picture of marked atrophy. Through these groups there was a further loss of spermatogenic cells, and prespermatogonia became hard to find. In the

2- and 4-month groups, prespermatogonia and spermatogonia exhibited a greater degree of depletion than did the other spermatogenic cell types. In the 4- and 6-month groups the Sertoli syncytium of the more severely damaged tubules filled the lumen and was vacuolated. In the 8-month group the syncytium appeared fibrotic, and pyknotic Sertoli-cell nuclei were seen in clusters in the centers of the tubules. From a just perceptible relative increase in the interstitial tissue in the 2-month group, there was a change to roughly equal amounts of interstitial tissue and tubular components in the 8-month group. The tubular basement membrane became thickened and wrinkled and the nuclei of its cells became swollen and shortened (fig. 1*C*). Cellular elements besides Sertoli cells were scarce within the tubules after 8 months of irradiation.

There was little further change in the 10- to 16-month groups over what was observed after 8 months' irradiation. Changes in the direction of even more severe degeneration were manifested by the absence of Sertoli cells along regions of tubular walls, and a further decrease in diameter of the tubules. In this period (10 to 16 months) almost always there were a few uninjured spermatogenic cells (up to 10) in a tubule, including some spermatogonia and prespermatogonia. The last-named cell types were rarely seen in sections from the testes of the 14- and 16-month groups. There was a relative increase in the presence of the basophilic stem cell in these last two groups. There was little further increase in the relative amount of interstitial tissue.

Degeneration of cells.—Apart from the changes of the Sertoli cells in the damaged tubules, degeneration attributable to radiation was not seen in other cellular types. Even in the normal testis there are abnormal and degenerative spermiogenic cells. In the testes of untreated mice and of those receiving 1.1 r daily, more degenerating and abnormal spermatids and spermia were seen than after treatment with 4.4 r and 8.8 r daily.

Globules in the nuclei of otherwise normal Sertoli cells occur rarely in the normal testis. A marked increase in such cells was taken to be an indication of early degeneration. In both the 4.4 r and 8.8 r series, the incidence of these globules was much higher than in the nonirradiated testes. The 1.1 r series did not differ from the untreated series in this respect. In the 4.4 r and 8.8 r series, the majority of the spermatogenic cells in anaphase and telaphase were atypical.

Interstitial Cells.—Throughout the treated series the interstitial tissue appeared to be unaffected. Its apparent increase with the larger accumulated doses of irradiation, in both the 4.4 r and 8.8 r series, was probably a relative increase, resulting from the depletion of the tubular constituents. Likewise, the thickening of the capsule, the wrinkling and thickening of the tubular basement membrane and the shortening and swelling of the nuclei of its cells, and the decrease in section size probably all resulted from the depletion of the tubular elements. These secondary changes were more marked with greater degree of cellular depletion within the tubules.

Connective-tissue mast cells were an extremely rare finding in the

interstitial tissue of both the treated and the untreated series. No leukocytic infiltration was observed in any of the sections examined; little debris was seen.

Summary.—Testes from mice subjected to whole-body irradiation with gamma radiation at a rate of 1.1 r daily (8 hours) did not differ histologically from testes of nonirradiated mice.

Testes from mice irradiated with 4.4 r daily (8 hours) showed slight degenerative changes up to 10 months of irradiation; there was an average of three scattered degenerate tubules per section (predominantly cross sections). Irradiation for 12 to 16 months caused a progressively increasing degeneration (about 10 degenerate tubules per section) and a slight generalized decrease in thickness of the tubular epithelium.

Irradiation at the rate of 8.8 r daily (8 hours) produced considerable generalized degeneration even after treatment for 2 months. There appeared to be a greater loss of spermatogonia and prespermatogonia than the other spermatogenic elements after irradiation for 2 to 4 months. Depletion progressed with continued irradiation and after 8 months almost all tubules were virtually devoid of cells other than Sertoli cells, some of which were degenerate. There was a relative increase in the basophilic stem cells after 14 and 16 months of irradiation.

Degenerating spermatogenic cells seen in the sections of irradiated mice could not be attributed to radiation, since more degenerating and abnormal spermatids and spermia were seen in testes of untreated animals and of those treated at the 1.1 r dose rate than in testes of those treated at the 4.4 r and 8.8 r dose rates.

The atrophic changes of the testes in response to chronic irradiation are considered to result from a depletion of the spermatogenic elements. This depletion causes a decrease in the size of the testis, decrease in tubular diameter, relative increase in interstitial tissue, thickening of the capsule, and thickening and wrinkling of the tubular basement membrane, with thickening and shortening of the nuclei of the cells of this membrane.

Degenerate Sertoli cells were seen in damaged tubules. In testes from mice belonging to the 4.4 r and 8.8 r series the nuclei of the Sertoli cells possessed a higher incidence of globules than did those from nonirradiated mice.

It is concluded that inhibition of spermatocytogenesis rather than destruction of spermatogenic elements plays the more important role in causing the depletion of the spermatogenic elements in the testis of the chronically irradiated mouse (see Discussion).

Ovary

The effects of radiation on the ovaries were qualitatively the same for all three dosage rates. Untreated animals at all stages normally have atretic follicles, including anovular primitive follicles. The appearance of small or great numbers of connective-tissue mast cells, arrangement of stromal spindle cells in cords, appearance of hyaline bodies, and amount of vascularity seemed to have no constant relation to either amount of

radiation or to age. Also, individual differences in sensitivity affected results somewhat, so that unless otherwise noted a statement for a given period is a summary of the animals at that period. The effects of the highest dosage rate will be described first and in greatest detail, since with a larger amount of radiation in a given time the changes due to radiation are less likely to be confused with changes due to increasing age.

Effects of 8.8 r daily (8 hours).—Two months:—There were no intact primitive ova, and there was an increase in number of anovular primitive follicles (rings). However, in the untreated controls the primitive ova were also decreased and rings were increased in number, but not to the same extent as in the irradiated animals. There was no decrease in the number of growing follicles although the number undergoing atresia was increased over the controls.

Four months.—There were no normal follicles at any stage of development, the ovaries being composed mostly of normal interstitial tissue. The controls had a few more atretic growing follicles and anovular primitive follicles than at 2 months.

TABLE 4.—*Main changes in ovaries after irradiation*

	Disappearance of normal primitive ova	Disappearance of normal growing follicles	Appearance of lutein cells other than in corpora lutea	Appearance of tubular downgrowths from germinal epithelium
	Months	*Months*	*Months*	*Months*
8.8 r	2	4	6	8
4.4 r	6	8	8	12
1.1 r	12	----	12	16
Unirradiated	16	----	14	----

Six months.—In this group there were no traces of larger follicles except for one animal that had one large atretic follicle and a primitive follicle, which was atretic but not anovular. At this time, particularly at the periphery of the ovary, the intestitial tissue contained many highly vacuolated cells distinguishable from typical lutein cells only by their arrangement in goups of irregular size and shape. Occasional small groups of lutein cells also had a ringlike arrangement and were intermixed with persistent "anovular rings." In these the cells were swollen and vacuolated, so that the two formations were sometimes hard to distinguish. Controls at this time had normal follicles of every type and contained lutein tissue only in well-defined corpora lutea.

Eight months.—There were no remnants of follicles of any sort, except for a few "rings" in one animal. Cords of cuboidal cells lined narrow spaces in the ovary in three of the animals. These were tubular downgrowths of the germinal epithelium, most numerous at the periphery and extending in a ramifying fashion through the ovary. Between these tubular structures the interstitial tissue in places was composed of lutein cells. Controls had many follicles of all types and lacked the downgrowth of epithelium seen in the irradiated animals.

Ten months.—The picture was much the same except that tubular downgrowths and lutein cells were increased in amount. Some of the lutein cells contained a green-staining pigment. Controls had follicles of all types, but with decreasing numbers, particularly of primitive ova.

Twelve to fourteen months.—The changes were progressive, tubular downgrowths occupying the greater part of the ovary. The interstitial cells were nearly all of the lutein type. For comparison, controls at 14 months had three to five normal growing follicles, two to three corpora lutea, but primitive ova had almost disappeared. The interstitial tissue had some small areas composed of lutein cells, and some of these had green pigment in them.

No sections of ovaries from animals at 16 months were available for study.

Effects of 4.4 r daily (8 hours).—At 2 months there were no histologic changes as compared with unirradiated animals. At 4 months there were almost no undamaged primitive ova, and there were more anovular primitive follicles than in the controls. From 6 months on there were no normal primitive ova in any animal. Normal growing follicles had all but disappeared. At this time one animal had some lutein interstitial tissue. At 8 months there were no normal growing follicles and even atretic follicles were scarce. There was an increased but variable amount of lutein interstitial tissue. Thus, one animal showed areas of highly vacuolated or moderately vacuolated cells as well as normal interstitial cells. At 10 months most of the ovarian tissue was composed of lutein cells, although there were some normal interstitial tissue and a few anovular primitive ova. At 12 months the amount of lutein tissue was increased, and some of the cells contained green pigment. In one animal there were beginning tubular downgrowths from the germinal epithelium. These changes continued, and at 14 to 16 months there was an overgrowth of germinal epithelium weaving around nests of lutein cells. There was a corpus luteum in one animal at the 16-month interval.

Effects of 1.1 r daily (8 hours).—Through the 10-month interval in the irradiated animals primitive follicles decreased while anovular primitive follicles and other signs of atresia increased. These changes, however, occurred also in the controls and to about the same extent, except that primitive ova were probably fewer than in controls from 6 months on.

At 12 months some of the interstitial tissue consisted of lutein cells, some with green pigment. Larger follicles were fewer than in controls, and primitive ova were all anovular, while controls usually had one or two normal ones per section.

At 14 to 16 months there were fewer normal, growing follicles than in controls, but there was one intact growing follicle even at the 16-month interval. Anovular rings were present but decreased, while lutein tissue was increased.

Ovarian Tumors After Irradiation

Serial sections of the ovary were not taken. No ovary of an irradiated animal in this series was larger than normal, except the few in which large

cysts occurred, and in these the amount of ovarian tissue was not increased.

Since the total amount of ovarian tissue was not increased, and since the increased amount of lutein tissue apparently progressed, not by expansion or infiltration, but by changes in the existing cells, it did not seem that either granulosa-cell tumor or luteoma was present in these animals. While the tubular downgrowths grow by replacement of tissue, this change is not regarded as a tubular adenoma until by its growth the size of the ovary is increased (*5*, *6*). This did not occur in these animals.

In acute-radiation experiments Furth and Butterworth (*5*) and Butterworth (*6*) have described certain ovarian tumors that are composed of tissue similar to some of the changed tissue seen in this experiment. Therefore, evidence of neoplastic change was looked for in this series. It would seem, however, that the changes described are best viewed as radiation-provoked degenerative changes, and that there was no true tumor formation. Presumably with more irradiation or with more time, tumors similar to those described by Furth and Butterworth might have developed.

The number of ovarian cysts in the treated mice was increased over that found in the controls. The majority were simple cysts but intracystic papillomata occurred in 4 animals, 3 in the 8.8 r group, and 1 in the 4.4 r group. These ovarian cysts are summarized in table 5.

Summary.—The changes in these ovaries were like those that occur in acute-radiation experiments and were qualitatively the same at the three-dose levels. The changes were progressive and, allowing for age changes and individual variation within the groups, seemed to depend upon the total amount of radiation (see table 4).

Primitive ova degenerated and disappeared, leaving small rings of follicular cells (anovular primitive follicles). Eventually, these too disappeared. Larger growing follicles became atretic and finally disappeared. These follicles were more radioresistant than the primitive ova in the mouse as pointed out by Schugt (*7*) and Murray (*8*).

Small nests of lutein cells appeared first at the periphery and later elsewhere in the ovary. These nests were often intermixed with changed anovular primitive follicles, so that it was sometimes difficult to distinguish the two. The amount of lutein tissue increased and some of its cells began to store progressive amounts of green-staining pigment.

Tubular downgrowths from the germinal epithelium appeared and grew irregularly through the entire ovary. Radiation in adequate quantity over a sufficiently long period produced ovaries with many ramifying tubular downgrowths separated by groups of lutein cells. This did not occur in any unirradiated animal, and the amount of downgrowth increased with more radiation.

Primitive ova disappeared in 2 months with 8.8 r, 6 months with 4.4 r, 12 months with 1.1 r, and 16 months in the unirradiated animals. Growing follicles had disappeared by 4 months with 8.8 r and 8 months with 4.4 r. Interstitial lutein cells appeared at 6 months with 8.8 r, 8 months

with 4.4 r, 12 months with 1.1 r, and at 14 months in the unirradiated animals. Tubular downgrowths appeared at 8 months with 8.8 r, 12 months with 4.4 r, and 16 months with 1.1 r.

In the mice receiving 1.1 r daily some follicles persisted, even after lutein cells began to form a part of the interstitial tissue; this was not true of the mice receiving 8.8 r and 4.4 r.

Irradiation, especially with 8.8 r daily, increased the number of cysts over those in the controls, but no other ovarian tumors developed (see table 5). No cysts occurred in 1.1 r animals.

Other Organs

In the gastrointestinal tract no change was consistently found in irradiated animals that was not seen in the controls, except the response of the Peyer's patches. These showed changes that paralleled the changes in the mesenteric lymph node. Of the 12 leukemic animals the Peyer's patches were involved in 3, and in 1 of them they showed the only leukemic change.

Table 5.—*Occurrence of ovarian cysts*

Dosage rate	Time sacrificed	Description
	Months	
0.0 r	12	Moderately large, lined by columnar cells.
0.0 r	14	Large, lined by columnar cells.
0.0 r	16	Hemorrhagic, lined by flattened stromal cells.
4.4 r	6	Small, lined by columnar cells.
4.4 r	8	Fairly large, lined by cuboidal to columnar cells.
4.4 r	12	Small, not lined but with regular outline.
4.4 r	12	Moderately large with beginning intracystic papilloma.
8.8 r	8	Early intracystic papilloma.
8.8 r	10	Very small cyst with no lining.
8.8 r	10	Large hemorrhagic, lined by stromal cells. Occurred in ovary with leukemic infiltration.
8.8 r	12	Large intracystic papilloma.
8.8 r	14	Intracystic papilloma.

One fibrosarcoma occurred in the upper portion of the small intestine.

The skin showed no gross or microscopic changes. There was no alopecia.

The liver, kidney, adrenal glands, pancreas, heart, and lungs showed no deviations from those of the control mice except for tumors and leukemic involvement as noted in tables 6 and 7.

Tumors and Leukemia

The effect of repeated exposure to low-intensity irradiation has been reported to cause the early appearance of spontaneously occurring tumors and lymphoma in LAF_1 mice [Lorenz *et al.* (*1*)].

The control animals in this experiment were sacrificed at 2-month intervals up to 16 months. The only spontaneous tumors in our control animals were adenomas of the lung. These resembled closely the bronchial adenomas described by Lippincott (*9*). They appeared to grow expansively; they were not encapsulated, being surrounded by compressed

lung tissue. Microscopically they consisted of closely packed columns of cuboidal or columnar cells arranged in a sparse stroma with few blood vessels. Metastasis was not seen. The bronchial epithelium showed small irregular thickenings that looked as though they might be possible forerunners of the adenomas. However, Grady (*10*) has reported that these tumors originate from alveoli and later invade the bronchus.

However, in addition to the bronchial adenomas, spontaneously occurring tumors reported by others during the full life span of this strain of mice also include hepatomas, breast carcinoma, and adenomas of the adrenal gland. Our control did not show these, but our treated animals did. The hepatomas have rounded noncapsulated margins surrounded by liver tissue. The tumors are made up of cells resembling hepatic parenchymal cells but show a wide variation in size. The lobular structure of the liver is lacking in the tumors. They do not appear malignant.

The incidence of lymphomas and of tumors (other than ovarian cysts) in our animals is summarized in tables 6 and 7, respectively. All of our groups had eight animals sacrificed at intervals of 2 months from 2 to 16 months. The groups not mentioned in the tables showed no lymphomas or tumors.

There was no lymphoma in the control animals. The lymphoma incidence for the entire life span of this strain is approximately 50 percent for females and 15 percent for males (*1*). In our experiment, all animals were sacrificed before or at 18 months. The incidence of leukemia increased in the irradiated animals in proportion to the intensity of irradiation. Of the 12 obvious leukemias in 192 irradiated animals, only 2 were in males. In addition, there were several animals in which there were marked hyperplastic changes in several hematopoietic organs. We have included as lymphomas only those on which two consulting pathologists agreed. From our material it was impossible to determine where the lymphoma first developed but from table 6 it appears that the thymus gland and Peyer's patches should be considered.

All three animals with myelogenous leukemia had extreme hyperplasia of the bone marrow with predominance of all stages of myeloid forms, marked enlargement of the spleen with accumulation of myeloid cells, and infiltration of organs and lymph nodes by myeloid cells in various stages of maturation (see table 6). Two of these animals had extremely high peripheral white-cell counts as demonstrated in cross section of the large vessels. These cells were predominantly of the mature myeloid type. The animal exposed for 14 months did not show the increase in cells in the peripheral blood and was probably an aleukemic leukemia.

Whereas 2 tumors were found in the controls, 4 were found in the 1.1 r series, 11 in the 4.4 r, and 10 in the 8.8 r. In both treated and untreated mice no tumors were found in groups earlier than 10 months. Of the 25 tumors found in the treated animals, 16 were in males, 9 in females. The lung adenomas and the hepatomas comprised the majority of the tumors. Five of the 10 treated mice with lung adenomas were males and 5 were females. On the other hand, 8 of the 10 hepatomas were in males, 2 in females.

TABLE 6.—*Organ involvement with leukemia*

Radiation	Time	Sex	Type lymphoma	Animal number	Organs involved								Lymph nodes
					Thymus gland	Spleen	Bone marrow	Liver	Intestine	Adrenal gland	Kidney	Pancreas	
	Months												
1.1 r	14	♀	Lymphatic	12820	0	+	0	0	0	+	+	+	
4.4 r	14	♀	do	12828	------	+	0	+	0	0	0	+	Mesenteric lymph nodes.
	14	♀	do	12829	0	0	0	0	+	------	------	0	
	16	♀	Myelogenous	24208	------	+	+	+	+	0	0	0	
8.8 r	10	♀	Stem cell	12765	------	+	+	------	------	0	0	0	
	12	♀	do	12836	+	+	------	+	0	0	+	------	Cervical and mediastinal lymph nodes.
	12	♀	Lymphatic	12798	+	0	+	0	0	0	0	0	Direct spread to fill mediastinum.
	14	♀	Myelogenous	24217	0	+	+	+	------	------	+	------	Cervical and mediastinal lymph nodes.
	14	♂	Lymphatic	12830	+	0	0	0	0	0	0	0	
	14	♂	do	12832	+	0	0	0	0	0	0	0	
	16	♀	do	24216	+	+	0	0	0	0	0	0	
	16	♀	Myelogenous	24218	0	+	+	+	+	------	+	+	Mass including intestinal mesenteric lymph node and pancreas.

TABLE 7.—*Incidence of tumors other than lymphoma or ovarian cysts*

	Time	Sex		Tumors
	Months			
Controls	10		F	1 lung adenoma.
	16	M		1 lung adenoma.
		1	1	2
1.1 r daily	10		F	1 lung adenoma.
	14	M		1 hepatoma.
	16	M		1 adenoma of sebaceous gland.
		M		1 hepatoma.
		3	1	4
4.4 r daily	12		F	1 fibrosarcoma of small intestine.*
		M		1 lung adenoma.
	14	M		1 hepatoma.
			F	1 lung adenoma.
	16	4M	2F	6 hepatomas.
			F	1 lung adenoma.
		6	5	11
8.8 r daily	10	2M		2 lung adenomas.
	12	M		1 hepatoma.
	14	M		1 adenoma of kidney.
		M		1 lung adenoma.
			F	1 adenocarcinoma of breast.
	16	M	2F	3 lung adenomas.
		M		1 adenoma of the sebaceous gland.
		7	3	10

*Died at 11 months, 9 days.

DISCUSSION

In these chronic experiments with low doses (1.1, 4.4, and 8.8 r) of daily irradiation with γ-radiation—accumulating to a maximum of 4,270 r—we have found changes in most, but not all, of the organs that are well-known to be susceptible to injury by a single, moderate-sized exposure of ionizing radiation. Thus, we found no changes in the gastrointestinal tract and skin. Unfortunately the eyes were not taken for histologic examination, and the bones had practically ceased growing before the exposures to gamma radiation were started. With our higher doses and exposure for long periods of time the other radiosensitive organs, specifically the hematopoietic organs and the sex glands, were damaged. There are several questions on the changes we found that merit some discussion, particularly changes in the numbers of mast cells and the details of testicular atrophy.

The mast cells are conspicuous in sections of mouse tissues fixed in Zenker-formol and stained with hematoxylin-eosin-azure II (see figs. 2 and 3). In several organs (thymus gland, spleen, bone marrow) there was a definite increase in connective-tissue mast cells with progressive amounts of irradiation. Fortunately there were adequate numbers of untreated control animals of both sexes, so that it was obvious the increase in mast cells was not a reflection of aging. It is noteworthy that mast cells were apparently decreased where there was leukemic involvement of the thymus gland, spleen, or bone marrow.

We have no explanation for either the decrease in these cells in leukemic animals or their increase after chronic irradiation. For these mice we have no data on changes in coagulability of the blood such as have been demonstrated for acute-radiation sickness. If an experiment such as the present one is ever repeated we think it desirable that tests on coagulation time should be made.

There is, in general, close agreement between the conclusions that may be drawn from our subjective microscopic observations on the testis and those made by Eschenbrenner, Miller, and Lorenz (*4*) after quantitative histologic analysis of companion experimental material. They report that the primary effect of chronic irradiation of the testis is on the spermatogenic elements of the organ, as is well known for acute irradiation. The interstitial tissue is unaffected. They state that the increase in the amount of interstitial tissue is a relative one, the absolute quantity of the tissue remaining the same. The decrease in weight and size of the testis, as well as the increase in capsule thickness, is the result of tubular atrophy. They believe that the effects of chronic irradiation on the mouse testis are not cumulative, the rate of irradiation being the significant factor.

There is, however, a point of disagreement. They report: "Despite marked decrease of total quantity of spermatogenic elements in the testes of irradiated mice, the proportion of these cells in different stages of spermatogenesis was normal in mice exposed to 1.1 r and 4.4 r daily for as long as 16 months. In mice exposed to 8.8 r daily, it was normal for 4 months, while after 6 or more months of irradiation, there was failure of completion of spermatogenesis, and this was associated with degeneration of Sertoli cells." We believe that the characteristic effect of irradiation after 2 and 4 months at the rate of 8.8 r daily was the greater depletion, in relation to the other cell types, of the dividing spermatogonia and prespermatogonia (the latter group includes the resting spermatogonia). Is it possible that our different conclusions, based on different methods of study, may be due to failure to account for the effect of the spermatogenic wave in both counts and qualitative observations made by random sampling of single sections? It would be good to have counts made on many serial sections.

We agree with their observation that failure of completion of spermatogenesis was associated with degeneration of Sertoli cells. This relationship was seen by us in the 8.8 r series after 6 or more months, and in individually affected tubules in the 4.4 r series.

It seems to us on the basis of the material reported here that there can be no question of the existence of the basophilic elongate cells located next to the tubular basement membrane. These cells show a nice series ranging in shape from elongate stem cells and prespermatogonia to round resting spermatogonia and they are shown in figures 1*A* and *B*. The observation of a relative increase in outstretched stem cells in the higher accumulated doses given at the rate of 8.8 r daily is interesting when one considers that the predominating remaining cells within the tubules were the Sertoli cells (fig. 1*C*). There is, however, not sufficient evidence to

permit one to say that the various basophilic elongate cells are but different forms of the same cell type, which might give rise to both Sertoli cells and spermatogonia.

In contrast to their previous position, Dr. Eschenbrenner and Miss Miller tell us in a personal communication (*11*) that they now feel that the Sertoli cells might arise from a cell which is the common precursor of both Sertoli cells and spermatogonia and that they do not arise from stromal cells.

With regard to whether chronic irradiation caused inhibition of spermatogenesis or destruction of the spermatogenic elements, the lack of debris leads us to believe that, if destruction did occur, it occurred at a very slow rate. The observation of a higher incidence of abnormal and degenerate spermatids and spermia in the nonirradiated testes and those irradiated at the 1.1 r dose rate than in those irradiated at the rate of 4.4 r or 8.8 r may well be interpreted to mean that spermatogenesis was proceeding at a faster rate in normal and low-dose groups. Accordingly, it may be tentatively concluded that inhibition of spermatocytogenesis rather than destruction of spermatogenic elements plays the more important role in causing the depletion of the spermatogenic elements in the chronically irradiated testes.

SUMMARY

Chronic effects of repeated low doses of external gamma radiation on LAF_1 mice were observed at three dose levels. The experimental animals, half male and half female, were divided into four groups; 1) untreated controls, 2) 1.1 r daily (8 hours), 3) 4.4 r daily (8 hours), and 4) 8.8 r daily (8 hours). In each group four males and four females were killed at bimonthly intervals from 2 to 16 months.

After 1.1 r daily (8 hours), only the ovary and thymus gland showed changes due to the irradiation. From 6 months on, primitive ova decreased more than in controls, with resulting changes in ovarian structure, and in the thymus gland an increase in mast cells and a decrease in lymphocytes began at 10 months.

After 4.4 r daily (8 hours), changes in the ovary and thymus gland occurred earlier and were more severe, and the testis, spleen, and bone marrow were also affected. Degenerative changes in the testis were slight until 10 months, increasing progressively at later intervals. Increase in mast cells began at 8 months in the thymus gland and spleen, and at 10 months in the bone marrow. The hematopoietic organs, with the exception of the lymph nodes, underwent progressive depletion, and at late intervals erythropoiesis increased in the spleen.

After 8.8 r daily (8 hours) the mesenteric lymph node was also susceptible to radiation injury, beginning at 10 months, in contrast to the response in the other hematopoietic organs, in which cellular depletion began much earlier. An increase in mast cells in the thymus gland, spleen, and bone marrow began earlier than in the mice exposed to 4.4 r daily (8 hours). Increased erythropoiesis in the spleen appeared at 6 months, in contrast to 14 months in the 4.4 r series. The proportion of

white pulp of the spleen also increased progressively. In bone marrow a shift toward more immature cells was detectable at 8 months.

Depletion of spermatogenesis was marked 2 months after irradiation, as were regressive changes in the ovary. Attempts at regeneration in the testis at 14 and 16 months were suggested by an increase in stem cells but there was no evidence of new formation of ova.

No explanation is offered for the great increase in the number of mast cells, which showed a good correlation with rate and size of dosage. Mast cells were decreased where lymphoma involved the thymus gland, spleen, or bone marrow.

Incidence of leukemia and of tumors was accelerated as a result of irradiation; it increased with the rate and time of exposure.

The peripheral blood showed mild anemia and definite lymphopenia in the later stages with 8.8 r daily.

The lowest dose used in these experiments [1.1 r daily (8 hours)] is clearly above the tolerance level of mice if continued for a sufficiently long time.

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PLATE 67

FIGURE 1.—*A:* Section of testis from untreated 10-month mouse, showing two prespermatogonia located against the basement membrane. Compare the prespermatogonia and stem cells in figures *B* and *C* with regard to shape, position, and basophilia. *B:* Section of testis of untreated 16-month mouse showing two elongate basophilic stem cells. *C:* Section of testis of 16-month mouse subjected to 8.8 r daily (8 hours) for 14 months showing a stem cell. Note the swollen nucleus of basement-membrane cell and the vacuole in Sertoli syncytium. Hematoxylin-eosin-azure II stain. × 1,250. Camera lucida drawing by J. R. Bloomfield.

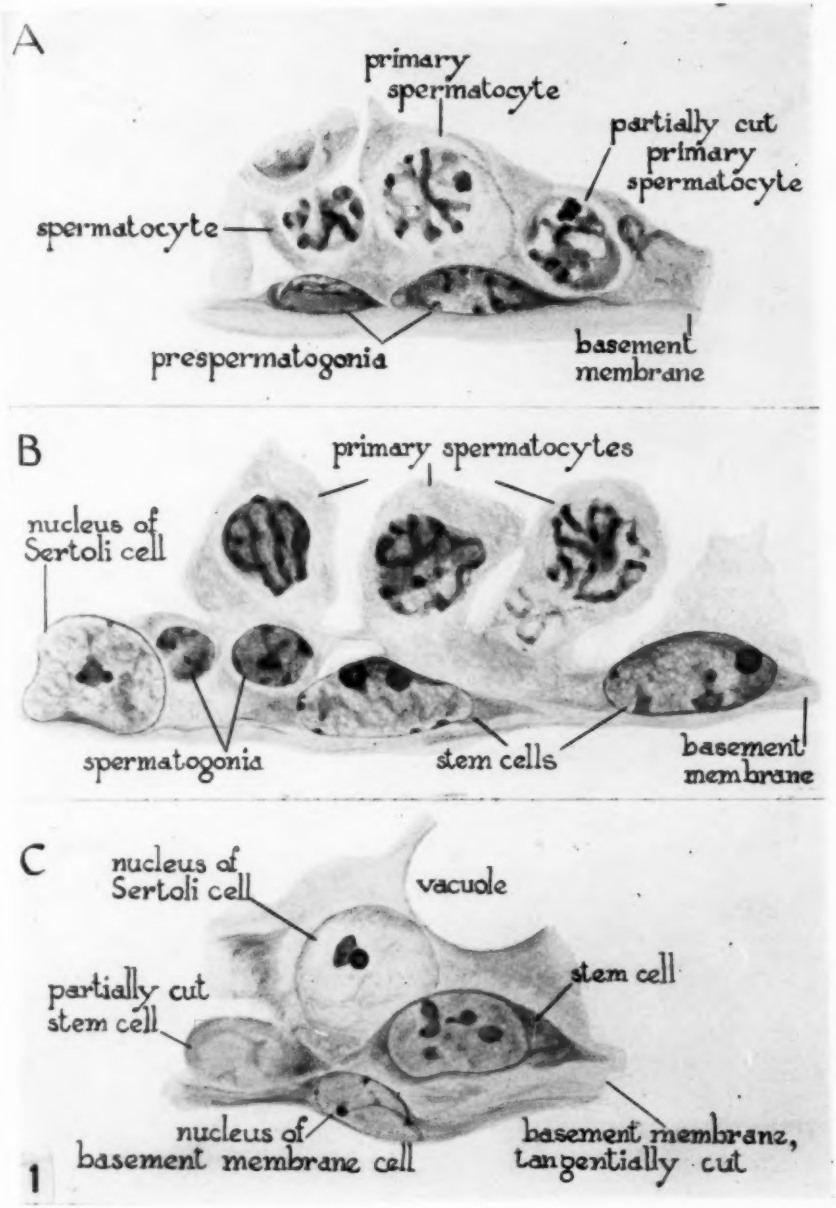
A
primary spermatocyte
partially cut primary spermatocyte
spermatocyte
prespermatogonia
basement membrane
B
primary spermatocytes
nucleus of Sertoli cell
spermatogonia
stem cells
basement membrane
C
nucleus of Sertoli cell
vacuole
stem cell
partially cut stem cell
nucleus of basement membrane cell
basement membranz, tangentially cut
1

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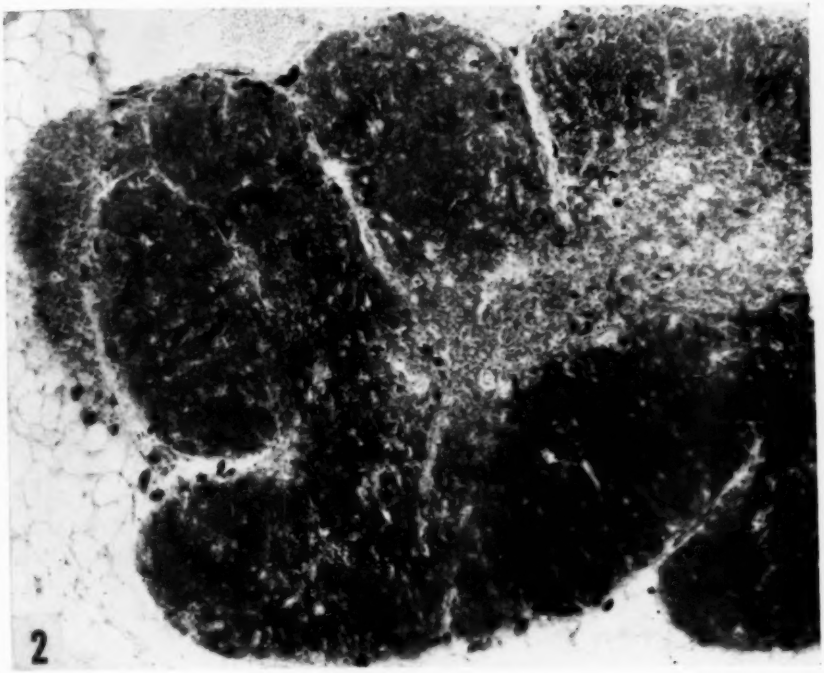
PLATE 68

FIGURE 2.—Section of thymus gland from female mouse irradiated with 4.4 r of external gamma radiation daily for 10 months. There is a mild depletion of lymphocytes and a small but definite increase in mast cells. Hematoxylin-eosin-azure II. × 160.

FIGURE 3.—Section of thymus gland from male mouse irradiated with 8.8 r external gamma radiation daily for 10 months. Note great depletion of lymphocytes and marked increase in number of mast cells. Hematoxylin-eosin-azure II. × 160.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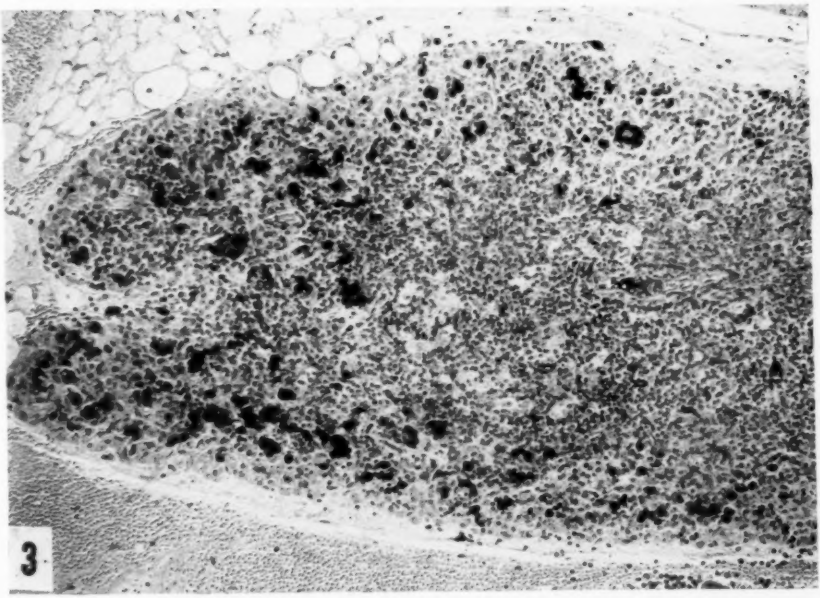
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PAPER PARTITION CHROMATOGRAPHY OF FREE AMINO ACIDS AND PEPTIDES OF NORMAL HUMAN GASTRIC JUICE [1] [2]

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INTRODUCTION

Published studies that reveal reliable information concerning either the total free or peptide amino-acid concentrations, or the identity or concentrations of the individual free or peptide amino acids in human gastric juice have not come to the present authors' attention.

In this investigation a technic of two-dimensional paper partition chromatography has been applied to gain information on the identity and concentrations of the individual free and peptide amino acids in the gastric juice of normal individuals. Chemical studies of the concentrations of protein, of the nonprotein nitrogen, and of the acidity of the gastric juices are also reported. Reference is made to our findings on analyses of normal human saliva and plasma by the same technic utilized in the study of the gastric juices.

This report affords background material for analysis and interpretation of the findings of studies by the same methods of the free and peptide amino acids of the gastric juice of patients with pathologic conditions of the stomach.

MATERIAL

Gastric juice was obtained from eight normal volunteers from four patients in the New England Deaconess Hospital who had no gastrointestinal or hepatic diseases.[4] Two of the specimens analyzed consisted of a pool of equal amounts of juice from two (#36) and from three (#39) normal volunteers. The gastric juice was aspirated, employing a Levin-tube aspirator, after the subjects had fasted overnight. From two normal volunteers, specimens (#23B and #25B) of gastric juice were also obtained after an alcohol meal: the alcohol meal, consisting of 100 ml. of 7 percent ethanol, was administered after withdrawal of the fasting juice, and the stomach contents were again aspirated 1 hour later. The material of the reported studies on plasma was obtained from the blood specimens, pre-

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[3] Gilligan: Biochemist, Laboratory of Pathology, Harvard Cancer Commission. Moor: Research Technician, Laboratory of Pathology, Harvard Cancer Commission. Warren: Pathologist, New England Deaconess Hospital.

[4] We are indebted to Miss Dorothy V. Blanchard, Supervisor of the Intravenous Therapy Department at the New England Deaconess Hospital, and to Dr. Bradley Copeland for their assistance in procuring the material of this investigation.

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vented from coagulating by heparin, of two normal volunteers after they had fasted overnight.

METHODS

Preparation of Specimens

The following procedures were carried out as promptly as feasible after the gastric juice had been aspirated. An aliquot of the gastric-juice specimen was removed for measurement of acidity; the pH was estimated with Alkacid Test Paper, with which a pH of less than 2 could not be estimated. The pH of the remainder of the juice, if acid, was immediately adjusted to approximately 7, according to Alkacid Test Paper, with minute amounts of 5 M NaOH. The juice was then filtered through gauze, followed by filtration through S. & S. #604 filter paper. An aliquot of filtered juice was taken for total nitrogen determination. The protein of the aliquot of filtered juice used for chromatographic and nonprotein-nitrogen studies was promptly precipitated with 95 percent ethanol in 10 times the volume of the aliquot and filtration was made after 10 minutes through #2 Whatman filter paper.[5] When dialysates of gastric juice were studied chromatographically the dialysates were obtained essentially in accord with the method of Hamilton and Archibald (*1*); dialysis was carried out in a cold room (t., +4° C.) and the dialyzing time varied from 4 to 24 hours. In the instances of specimens #38 and #39 dialysis was allowed to continue for 24 hours against a 0.02 M citrate buffer of pH 5.0 (at which pH both pepsin and trypsin reputedly have little or no proteolytic activity).

The blood samples were centrifuged promptly after their withdrawal and the plasma was removed. Removal of protein from the plasma utilized for chromatographic studies was accomplished both by ethanol and dialysis (against water for 4 hours in the cold room) as described above.

Samples of the alcohol filtrates of the gastric juice were further prepared for chromatographic study by the following methods. In some instances an aliquot of 22 ml. of filtrate, equivalent to 2 ml. of gastric juice, was transferred to a porcelain evaporating dish and evaporated just, or almost, to dryness over a *boiling water bath*. By serial washings with small amounts of water the contents of the evaporating dish were transferred, with a Folin micropipette calibrated to contain 0.2 ml., to a small test tube and to a total volume of 0.2 ml., *i. e.*, a volume one-tenth that of the 2 ml. of juice.[6] At other times the volume of the evaporated alcohol filtrate was brought to 2.0 ml., equivalent to the original volume of the gastric juice employed. These preparations were slightly cloudy and had, sometimes, a very slight yellowish coloration; care was exercised to mix all preparations thoroughly before "spotting" on the paper amounts equivalent to 125 or 250 μl. of the juice for chromatographic study. In other instances aliquots of the alcohol filtrates of gastric juice and of the dialysates of

[5] Precipitation of the protein of gastric juice by alcohol should not be attempted unless the pH of the juice has been adjusted to approximately 7.0, for otherwise a clear alcoholic filtrate may not be obtained.

[6] Alcohol filtrates of plasma when concentrated in this manner to one-tenth of the original volume of plasma are always very cloudy and contain unhomogenized lipids. We do not advise this method of preparation of plasma as we have obtained lower results on such concentrates than on material prepared as follows.

the juice (equivalent to 125 μl. of the juice) were transferred to small containers and evaporated to minute volume by a stream of air at room temperature and spotted on the paper with serial washings of the container.

Acid hydrolysis was effected in essentially the same manner as employed by Dent and Schilling (*2*) or, when larger volumes of solution were hydrolyzed this was accomplished in a 6 N concentration of HCl in an Erlenmeyer flask covered with a glass marble in an autoclave at 15 pounds pressure for 18 hours. After evaporating the hydrolysate to dryness and repeating this procedure several times after redissolving in water, the solution was finally neutralized by the addition of alkali before washing onto the paper for chromatographic analysis.

Qualitative Chromatographic Studies

Preliminary studies were made on one-dimensional chromatograms with phenol, and with collidine-lutidine as solvents. There was so much overlapping of the many amino acids present in gastric juice that two-dimensional analyses by the ascending method, using "Pyrex" jars and Whatman #1 filter paper, described by Williams and Kirby (*3*) were later employed exclusively.

Phenol (Merck) saturated with water was used as the first solvent (*4*) followed by a mixture of collidine and lutidine, also saturated with water, as second solvent (*4*). Refined grades of 2,4,6-collidine and 2,4-lutidine obtained in 1-gallon cans from Koppers Co., Inc.[7] were satisfactory. We found that with these solvents a mixture containing 25 parts of collidine and 75 parts of lutidine, saturated with water, gave a much better spread of amino acids than a 50-50 mixture, and R_f values practically identical with those found by Dent (*4*) in the 50-50 mixture of his solvents. To avoid droplets of water we have filtered the solvents through filter paper before transferring them to the tanks (*5*).

Chromatograms of the unhydrolyzed and hydrolyzed specimens were started in their first solvent as soon as they were prepared for analysis.

The papers were run in both solvents overnight, the phenol running about 16–18 hours and the faster moving "collidine" [8] running about 15 hours. After the phenol run, the papers were dried for some 6 hours at room temperature in the hood with a flow of air, then turned and run that night in the "collidine," following which they were dried again in the same way for at least 2 hours before spraying with ninhydrin. After spraying with a solution of 0.25 percent ninhydrin in n-butanol saturated with water (*3*) the papers were again dried as above for 30–60 minutes and placed in an oven at 90° C. for 15 minutes. The spots were outlined, and their intensities estimated (see below), on viewing with transmitted light from an X-Ray Circline Illuminator, on the same day of spraying.

Maps of knowns were made from solutions of the amino acids prepared essentially as described by Dent (*4*). Most of the amino acids were dis-

[7] Koppers Co., Inc., Tar Products Division, Pittsburgh 19, Pa. A 25-75 mixture from new cans of collidine and lutidine obtained from Koppers Co., Inc., some 6 months after our first order gave the same spread as obtained from our original supply.

[8] We have used the abbreviation "collidine" in the text as referring to mixtures of collidine and lutidine.

solved in 75 percent ethanol; a few compounds were dissolved in water. In accordance with the observations of others, and of ourselves, that the color (*6*) of the ninhydrin compound of an amino acid and the position the amino acid takes on a chromatogram (personal observations) (*7–9*) may differ with the pH at which it is spotted onto the paper, we have neutralized our solutions with the exception of those few substances that were extremely insoluble at pH 7.0.

Because on our chromatograms of the body fluids studied there was uniformly a large yellow "salt spot" [(*2*, *10*); (text-fig. 1, in the position of R_f values of approximately 0.16 and 0.24 in phenol and "collidine," respectively)], which distorted the shape and position of certain amino acids, we have studied maps of knowns both without and with sodium chloride (in amounts of 12.5 and 25μl. of 8 percent solution) added to the paper before running the chromatograms. The "salt spot" obtained from these additions of NaCl to a mixture of known amino acids was yellow in color and identical in position with the above described "salt spot" observed on the chromatograms of body fluids. Similarly, the distortions of the amino acids by this "salt spot" were the same on the map of knowns as on the chromatograms of the body fluids. In addition, we have noted occasionally on chromatograms of body fluids smaller yellow "salt spots" in the position of R_f values of approximately 0.45 and 0.18, and 0.65 and 0.18 in phenol and "collidine," respectively. These latter spots did not appear to cause troublesome distortions. The nature and positions on chromatograms of "salt spots" seen in analyses of un-"desalted" body fluids have been discussed by Dent *et al.* (*2*) and by Westall (*10*).

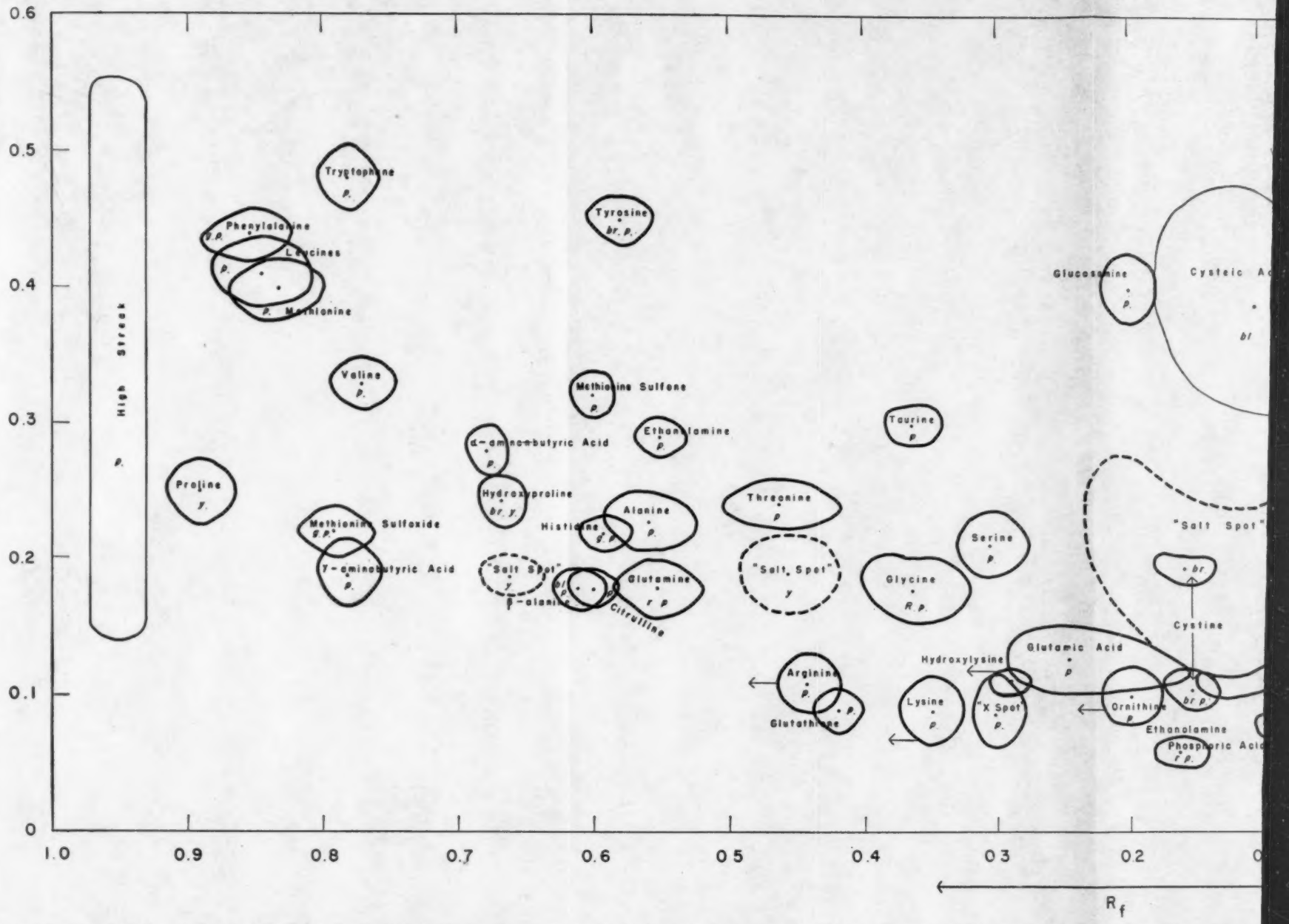
The map presented (text-fig. 1) is a composite constructed from the spot maps obtained on knowns, and the spot maps of identified and unidentified ninhydrin-reacting substances obtained on the chromatograms of body fluids and hydrolysates of protein-free body fluids.

For the detection of methionine and cystine, specimens have been treated on additional papers before running the chromatograms by addition of H_2O_2 and ammonium molybdate (*4*). The method of Work (*11*) for separating the leucines, phenylalanine, and methionine was also employed in the study of a few gastric juices, the solvent (*11*) being allowed to descend for 5 days on the paper in a Chromatocab.

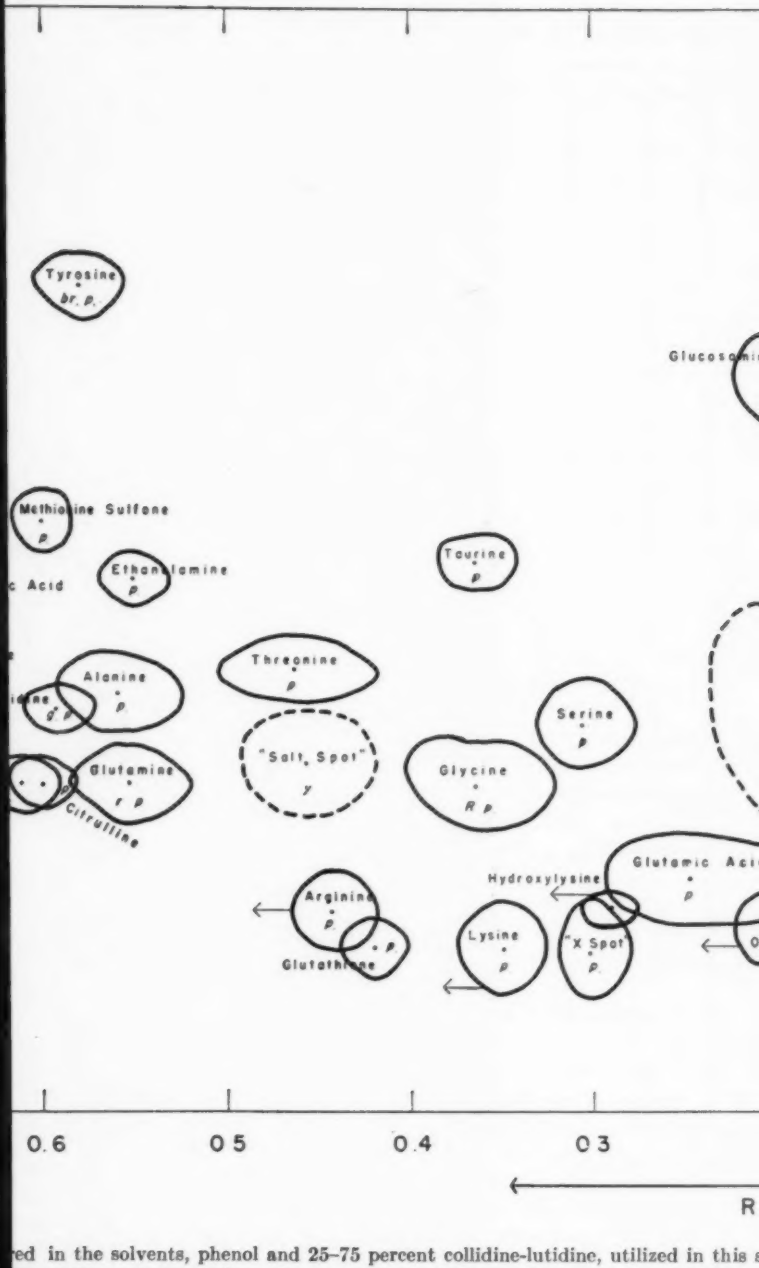
Although phenylalanine overlaps the leucines and methionine in the phenol-"collidine" solvents, our experiences have been that its position and purplish gray color are sufficiently different from these other amino acids to allow identification of its presence in appreciable amounts.

Positive identification of several of the amino acids has been made by adding knowns to spots of gastric juice or plasma on the papers of additional chromatograms; thus, when γ-aminobutyric acid,[9] arginine, aspartic acid, glutamine, lysine, and proline were added no new spots appeared, and these substances exactly superimposed on the chromatogram the spots believed due to their presence in the body fluids. Since methionine

[9] We are indebted to Dr. C. E. Dent for a sample of γ-aminobutyric acid, to Dr. James Weisiger and Dr. D. D. Van Slyke for one of hydroxylysine, and to Dr. F. Lee Rodkey for one of cysteic acid.



TEXT-FIGURE 1.—Map of spots of ninhydrin-reacting substances as they occurred in the solvents, phenol and 25–75 percent collidine-lutidine, utilized in this study. The map was obtained on knowns and the spot maps of identified and unidentified ninhydrin-reacting substances from the many chromatograms obtained on body fluids and hydrolysates. The on the map as follows: p. purple, g. gray, br. brown, y. yellow, bl. blue, and r. red, R. more red than r. The average solvent fronts were 25 cm. and 32 cm. in phenol and "collidi



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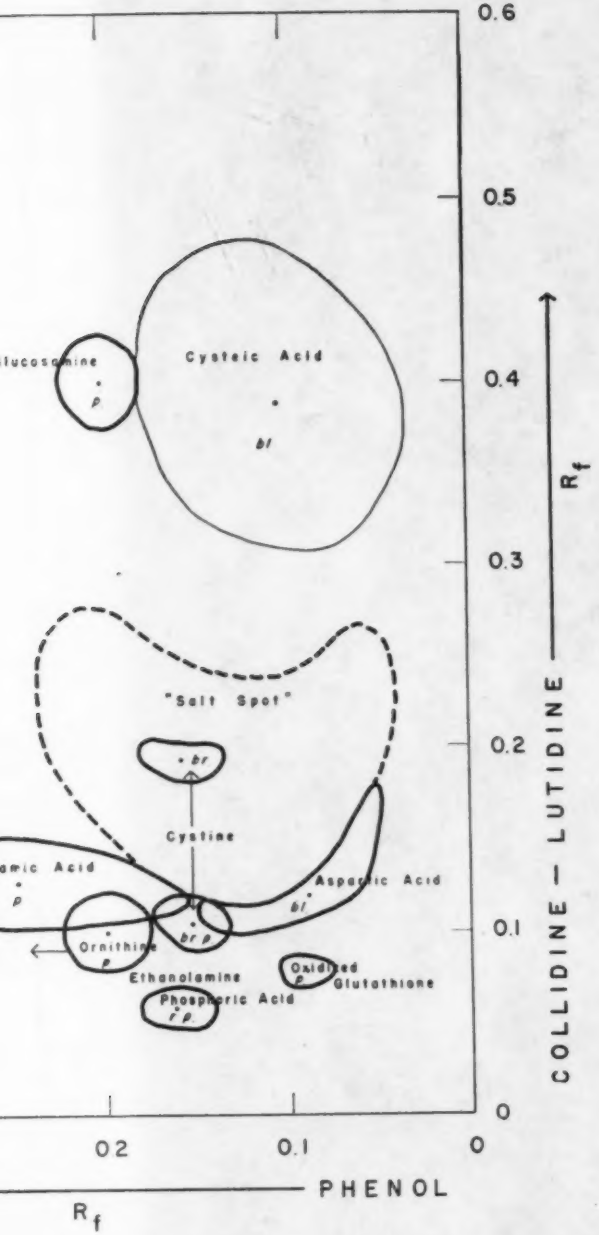

d in this study. The map was constructed from the spot maps
y fluids and hydrolysates. The colors of the spots are recorded
nd 32 cm. in phenol and "collidine," respectively.

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sulfoxide and γ-aminobutyric acid overlap each other somewhat on our chromatograms (text-fig. 1) methionine sulfoxide [from methionine plus H_2O_2(*12*)] was added on the paper to gastric-juice filtrates, the original chromatograms of which showed spots in the region of, and interpreted to be due to, γ-aminobutyric acid. The finished chromatograms showed the added methionine sulfoxide with its grayish-blue color somewhat overlapping but not superimposed on the γ-aminobutyric acid (text-fig. 1), which was easily distinguishable from it.

We became suspicious during study of our chromatograms of unknowns that lysine and arginine might under some conditions take positions higher in phenol than those found on our studies of knowns. By spotting these basic amino acids, and ornithine, in solutions of varying pH on the paper, we found that the R_f values of these substances in phenol tended to increase as the alkalinity of the solutions increased, and that values up to R_f's of approximately 0.70 in phenol were obtained with very alkaline solutions; also, hydroxylysine streaked markedly up in phenol when spotted on in the alkaline range. These findings confirm publications (*7-9*) that appeared subsequent to our studies, demonstrating that the above-investigated and certain other amino acids take varying positions on the phenol-collidine map (and in other solvents) dependent upon the pH of the "run."

In the absence of salt, cystine, cysteine,[10] and cysteic acid are always well separated from aspartic acid. However, when sodium chloride is added on the paper, cystine [cysteine(?)] is "shouldered off" to some extent by the low "salt spot" (text-fig. 1). Similarly, cysteic acid is in part "shouldered off" by the low "salt spot" and held in the position shown for aspartic acid (text-fig. 1). The difference in the colors of the aspartic acid (blue) and of any cysteic acid that has been "shouldered off" (purple) is of aid in identifying the substances. Since in our chromatograms of unknowns the color of the spot in question was always the blue of aspartic acid, we have recorded the spot as being due to this acid. Lack of success in identification and/or quantitation of cystine, cysteine, and cysteic acid in body fluids by paper chromatographic methods has been realized by others; Dent (*4*) reported that cystine and cysteine are usually not seen (at least when only a few μg. are present) on two-dimensional chromatograms with phenol followed by "collidine."

Quantitative Estimations From Chromatographic Studies

The sizes in $cm.^2$ of the spots on the chromatograms were measured with a Keuffel and Esser compensating polar planimeter.

The intensity of the purple spots on the chromatograms was measured against purple spots of varying intensity on a chart, prepared from a mixture of water colors which gave a purple color with transmitted light similar to the purple color given by the large majority of the amino acids when treated with ninhydrin on the papers. The mixture of water colors was

[10] When a fresh neutral solution of cysteine has been spotted on the paper the finished chromatogram shows spots identical to those of cysteic acid, indicating oxidation of the cysteine.

diluted serially from a concentration designated as 100 percent intensity down to 2.5 percent, to obtain mixtures of 2.5, 5, 10, 25, 50, 75, and 100 percent intensity. A 0.1-ml. amount of each of these dilutions was transferred with a small paint brush, and using a circular stroke, to circles 2.5 cm. in diameter outlined on No. 1 Whatman filter paper. Each spot thus obtained was of relatively uniform intensity throughout. (The intensity of the spot for 2.5 percent was approximately that of the limit of detectability of a ninhydrin-reacting substance on the chromatograms.) We have adhered to the practice of outlining the spots on the chromatograms just at their visible edges. Since the spots on a chromatogram are not of uniform intensity, being weaker on the periphery, it was necessary for comparison with the color chart to judge their average over-all intensity. Despite the distinctly reddish-purple color of glycine, we have made estimations of its intensity against the color chart. Yellow spots, such as given by proline, the blue spot of cysteic acid and the dull purple spot of tyrosine (the latter, in our earlier studies) have been estimated arbitrarily as weak, medium, and strong.

For most of the amino acids detected in gastric juice, standards of five different concentrations from 10 to 100 percent have been chromatographed repeatedly; the intensities of the spots obtained have been compared with the color chart exactly as have the unknowns, and the sizes of the spots have been measured. The amount of amino acid used for the 100 percent known was chosen to give a spot of intensity comparable on the color chart to an intensity between 25 and 50 percent. Curves have been drawn relating the intensities as measured against the color chart of the five different dilutions of these knowns to the amount of amino acid present, relating the sizes of the spots against the amount of amino acid present, and also relating the size times the intensities of the spots against the amount of amino acid present. Although it was obvious that in general the amount of an amino acid could be very roughly estimated from a chromatogram by comparing the intensity of the spot alone against the standard curve, or the size of the spot alone against the standard curve, it was also evident that greater accuracy was obtained when both the size of the spot and its intensity were considered. This approach afforded a means toward compensation for uncontrollable variations in the size of spots, such as occur when a spot is streaky or is crowded or distorted by salt, as has been demonstrated by runs of knowns to which sodium chloride has been added. Relating these two measurements of size and intensity by obtaining a "factor" of size multiplied by intensity gave good curves for dilutions of the various standards which were reproducible within approximately ± 20 percent on different runs made throughout the course of the study reported. Over the range of detectability up to a "factor" of approximately 150 these curves were straight line and went to the origin. Detectability failed at varying points above the origin. Above a "factor" of 150, the "factor" increased to a greater extent for a given increment of the amount of amino acid, and the values became less reliable. The number of μg. of the different amino acids that were found to give a

"factor" of 100 were as follows: alanine 2.1, γ-aminobutyric acid 3.5, arginine 15.0, aspartic acid 3.0, glutamic acid 3.4, glutamine 7.1, glycine 2.6, isoleucine 4.1, l-leucine 4.6, lysine 6.8, taurine 5.3, tyrosine 7.0, valine 3.7, serine 6.0, and threonine 11.4. From these values, and the "factors" found for a detectable amino acid in an unknown amount, by taking into consideration the amount of body fluid "spotted on" and the α-NH_2 N in accord with the molecular weight of a given amino acid, the chromatographic analyses were calculated in terms of α-NH_2 N concentrations.[11] The α-NH_2 N of the "leucines" has been calculated on the basis of the average value between isoleucine and l-leucine (see Results).

Whenever the measured size times intensity of a spot was greater than 150 this has been designated on the tables by italicizing the α-NH_2 N value given, to indicate that this figure represents a less accurate and somewhat too high value in comparison with the figures not underlined. Similarly any totals gained from additions of amino acids among which there appear underlined values have been underlined on the table.

Since there were not found in the literature data concerning the individual amino-acid constituents of gastric juice, the above methods of chromatographic study have been tested by comparing the results so attained for two specimens of normal human plasma with reported results for plasma obtained by chemical and microbiological methods. This study demonstrated that many of the amino acids present in small concentrations in plasma are not detectable on chromatograms of 125 μl. amounts (table 1). Further, the concentrations of those amino acids that were detectable on chromatograms of plasma could be estimated with an informative degree of accuracy (table 1). Roughly two-thirds of the total α-NH_2 N of the plasma was detectable (table 1) in the amounts of fluid (equivalent to 125 μl. of plasma) chromatographed (see Discussion).

Chemical Methods

The total nitrogen concentrations of the gastric juices after filtration through gauze and #604 S. & S. filter paper, were measured in duplicate on samples of 0.5 ml. by the micro-Kjeldahl technic of Ma and Zuazaga (*22*).

The nonprotein-nitrogen concentrations of aliquot samples of the same dialysates and alcohol filtrates used for the chromatographic studies were measured (after evaporation of the alcohol from the filtrates in the presence of a drop of H_2SO_4) by the same technic (*22*).

The protein concentrations of the gastric juices were calculated by subtracting the nonprotein nitrogen from the total nitrogen values and multiplying this value, expressed in grams per 100 ml., by 6.25. It is to be noted, however, that the mucoprotein of the gastric secretion elicited by sham feeding in the dog has a nitrogen content of about 14 percent (*23*),

[11] For simplicity of reporting, and in accord with practices of previous authors, γ-aminobutyric acid and proline are reported as if their ninhydrin color reactions were due to α-amino-nitrogen linkages, and glutathione as if it were an amino acid rather than a peptide. The terms "peptide" and "peptide nitrogen" are used throughout this report to denote amino-acid conjugates (supposedly other than protein) which yielded free amino acids on hydrolysis, although such substances as -acyl derivatives of amino acids, for instance, hippuric acid, may be included (*2, 15*). The term "detectable" is utilized to connote that a spot for a compound was detectable in the amount of fluid spotted on the paper.

TABLE 1.—*Average concentrations of the detectable amino acids of the dialysates of plasma from two normal fasting individuals as estimated from chromatograms, compared with the average concentrations reported in the literature for normal plasmas as gained from chemical and microbiological studies. (All values expressed as mg. of α-amino nitrogen (see Methods) per 100 ml. of plasma)*

Amino acid	Chromatographic estimation	Values reported in literature	Reference numbers
α-Alanine	0. 33	0. 53, 0. 63	(*14, 15*)
Arginine	[1] .	0. 18	(*16*)
Aspartic acid	0. 03	N. P.[1]	(*17*)
Cysteic acid (from cystine)	[1] W	N. P	(*17*)
Glutamic acid	0. 05	0. 32, 0. 10	(*18, 19*)
Glutamine	0. 63	0. 57, 0. 69	(*18, 19*)
Glycine	0. 45	0. 32, 0. 33	(*14, 15*)
Histidine	.	0. 13	(*16*)
Leucines (iso- and l-)	0. 47	0. 37	(*16*)
Lysine	0. 08	0. 29	(*16*)
Methionine	.	0. 05, 0. 06	(*20, 21*)
Phenylalanine	.	0. 12	(*16*)
Proline	M	N. P	(*17*)
Threonine	.	0. 24	(*16*)
Tryptophane	.	0. 12	(*16*)
Tyrosine	0. 04	0. 12	(*16*)
Valine	0. 37	0. 34	(*16*)
Total	[2] 2. 5+	3. 7+	

[1] A dot (.) signifies that the amino acid was not detectable on chromatograms of the plasma dialysates equivalent to 125 μl. of plasma; "N. P." signifies that the concentration of the amino acid has been cited to constitute only a negligible portion of the total free amino acids of plasma; "W" and "M" signify weak and medium intensit y of color, respectively.

[2] The average total detectable free α-amino nitrogen estimated from chromatograms of alcohol filtrates of these same two plasma specimens was 2.3+ mg. per 100 ml., and the average total α-amino nitrogen after hydrolysis of these filtrates was 3.2 mg. per 100 ml.

so that this factor of 6.25 is probably somewhat low (against 7.1) for calculating the mucoprotein constituent of the human gastric juice and saliva.

RESULTS

Spots showing the presence of alanine, aspartic acid, glycine, the leucines,[12] and valine were detectable on the chromatograms of the unhydrolyzed alcohol filtrates and dialysates of all of the nine specimens of gastric juice from fasting normal individuals; in addition, spots for γ-aminobutyric acid, glutamic acid,[13] glutamine, tyrosine and a "high streak" were usually present (table 2, text-fig. 1). "Weak" spots for other amino acids were less frequently observed (table 2), and a weak spot for an additional unidentified ninhydrin-reacting substance ["X" spot, (text-fig. 1)] was observed in one instance. The estimated α-NH_2N concentration of the leucines was uniformly the highest of all of the amino acids; the leucines, valine, and alanine together comprised two-thirds of the estimated total detectable α-NH_2N of normal gastric juice. There was considerable variation in the concentrations of α-NH_2N of a given detectable amino acid in the different specimens of gastric juice. However, the over-all "patterns" of the chromatograms from different speci-

[12] The chromatographic method of Work (*11*) for separation of the leucines was utilized in the further study of four gastric juice specimens. The presence in these juices of both isoleucine and l-leucine was demonstrated.

[13] It is possible that the small amount of glutamic acid found on the chromatograms of gastric juice may have been derived from glutamine by hydrolysis in the stomach.

mens showed similarity of character. The free amino acids of the alcohol filtrates and dialysates of the same specimen were alike, save in specimen #36 (table 2). The average total concentration of detectable free α-amino nitrogen was 0.9 +mg. per 100 ml., with limits of 0.6+ to 1.4+ (Alcohol filtrates, table 2).

The chromatograms of the hydrolyzed alcohol filtrates and dialysates of the gastric juices showed the presence of many more amino acids and very much higher concentrations of amino acids than were observed on the chromatograms of the unhydrolyzed specimens. The average total concentration of detectable α-amino nitrogen after hydrolysis of the alcohol filtrates was *12.8* mg. per 100 ml., compared to 0.9+mg. per 100 ml. before hydrolysis. Alanine, aspartic acid, glutamic acid, glycine, the leucines, and valine were estimated to be greatly increased by hydrolysis; certain other amino acids were also found in increased amounts after hydrolysis, and others appeared only on the chromatograms of the hydrolyzed specimens (table 2).

There was present frequently on the chromatograms of the unhydrolyzed specimens of gastric juice a pale purple streak, (the "high streak") with an average R_f in phenol of 0.95 and streaking in "collidine" over the R_f range of approximately 0.15–0.55 (text-fig. 1, table 2). This streak always disappeared on hydrolysis. In several instances elution (*12*) of the substances in the position of this streak from one-dimensional chromatograms in phenol or from two-dimensional chromatograms in phenol and "collidine" was made, and the eluate hydrolyzed. Chromatograms run on these hydrolyzed eluates repeatedly showed strong spots for many amino acids, commonly present in protein, including alanine, aspartic acid, glutamic acid, glycine, the leucines, proline, serine, threonine, tyrosine, and valine. Further attempts to identify this streak were beyond the scope of the present work.

An unidentified "weak" spot (text-fig. 1 "X–Spot"), which disappeared on hydrolysis, was seen, at R_f values of approximately 0.30 and 0.09 in phenol and "collidine" respectively, on the chromatogram of one normal gastric juice (table 2).

The chromatogram of the free amino acids of the gastric juice aspirated from one normal individual 1 hour after the alcohol meal was not strikingly different from that of the fasting juice (table 3, Specimens #25A and 25B). In another normal individual the chromatogram of the free amino acids after the alcohol meal was very much "weaker" than that for the fasting juice (table 3, Specimens #23A and 23B). The chromatograms of the hydrolyzed alcohol filtrates of the juices from both of these individuals showed much lower concentrations of detectable α–NH_2N after the alcohol meal than were present in the fasting juices (table 3).

Comparison of the chromatograms of fasting gastric juice with those of plasma revealed (as was expected from the fact that the former body fluid is a composite of secretions and the latter an extracellular fluid) great differences in the free and total amino-acid patterns of these fluids (text-fig. 2). For instance, the concentrations of glutamine, alanine, valine,

TABLE 2.—*Concentrations of free and combined amino acids in the gastric juice of normal fasting subjects detectable on, and estimated from, chromatograms* [1] *(amino acid values are expressed in mg. per 100 ml. of α-amino nitrogen)*

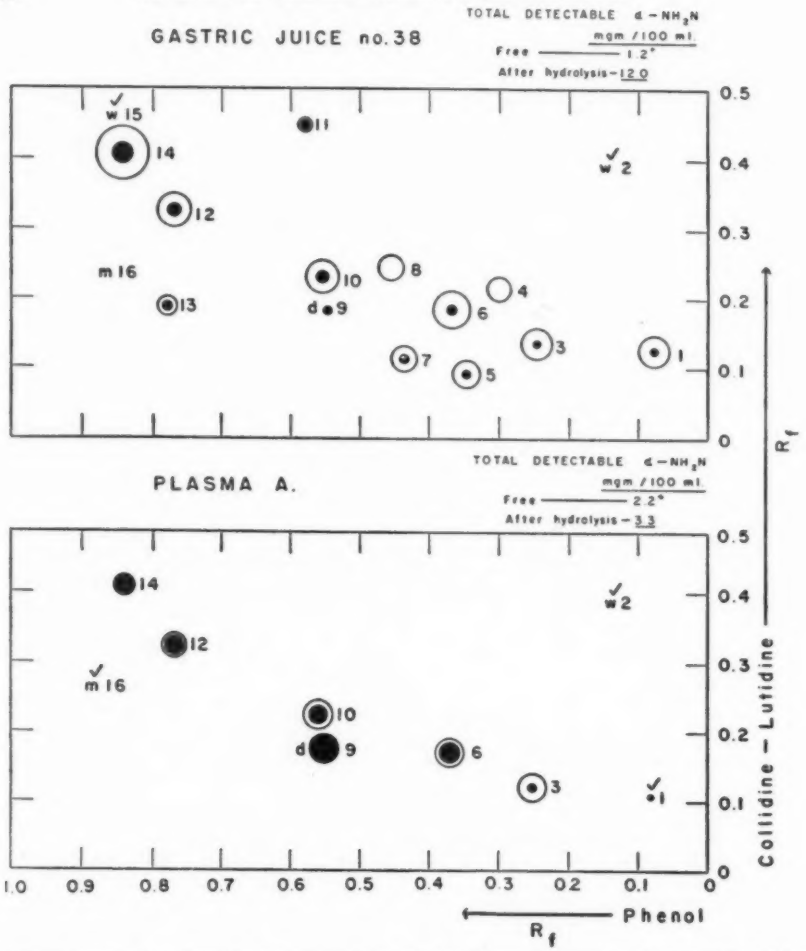
Gastric juice specimen	#39				#38				#36				#26		#25	
Sample analyzed	Alcohol filtrate	Alcohol filtrate hydrolyzed	Dialysate	Dialysate hydrolyzed	Alcohol filtrate	Alcohol filtrate hydrolyzed	Dialysate	Dialysate hydrolyzed	Alcohol filtrate	Alcohol filtrate hydrolyzed	Dialysate	Dialysate hydrolyzed	Alcohol filtrate	Alcohol filtrate hydrolyzed	Alcohol filtrate	Alcohol filtrate hydrolyzed
Alanine	0. 10	1. 46	0. 08	1. 46	0. 13	1. 36	0. 13	1. 17	0. 12	0. 53	0. 31	0. 64	0. 10	*1. 74*	0. 04	*1. 09*
γ-aminobutyric acid	0. 35	0. 15	0. 21	0. 30	0. 18	0. 23	0. 08	0. 30	0. 18	0. 18	0. 11	0. 20	0. 02	0. 15	0. 11	0. 13
Arginine	0. 13	0. 48	0. 14	0. 63	.	.	0. 05	0. 62	.	0. 39	0. 66	0. 81	.	1. 16	.	.
Aspartic acid	0. 02	*1. 55*	0. 04	1. 29	0. 03	0. 43	0. 03	1. 00	0. 03	0. 74	0. 12	0. 77	0. 05	1. 45	0. 01	*1. 78*
Cysteic acid (from cystine)	.	W	W	W	.	.	W	W	W	W	W	W	.	.	.	.
Glutamic acid	0. 05	*1. 66*	0. 03	1. 33	0. 03	0. 87	0. 03	0. 98	0. 03	1. 54	0. 07	1. 49	0. 02	*2. 02*	.	*1. 82*
Glutamine	0. 11	.	0. 14	.	0. 12	.	0. 06	.	0. 05	.	0. 35	.	0. 07	.	0. 04	.
Glutathione	.	.	.	.	.	.	.	.	.	.	.	.	W	.	.	.
Glutathione oxidized	W	.	W	.	.	.	.	.	.	.	W	.	W	.	W	.
Glycine	0. 09	*2. 52*	0. 13	*2. 73*	0. 04	1. 56	0. 06	1. 45	0. 06	*2. 52*	0. 25	1. 92	0. 05	*2. 32*	0. 03	*1. 74*
Histidine	?	?	?	?	?	?	?	?	?	?	?	?	?	.	?	.
Leucines	0. 39	*2. 68*	0. 44	2. 20	0. 37	*2. 37*	0. 46	*2. 94*	0. 41	1. 02	*1. 27*	1. 35	0. 45	*4. 02*	0. 24	*2. 54*
Lysine	.	0. 47	.	0. 63	0. 05	0. 52	0. 06	0. 81	.	0. 29	.	0. 23	0. 11	0. 29	.	0. 22
Methionine sulfone	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
Phenylalanine	W	.	.	.	W	M	W	W	.	.	.	.	W	S	W	W
Proline	.	W	.	M	.	.	.	W	.	M	.	M	.	S	.	S
Serine	.	1. 08	.	1. 33	.	0. 34	.	0. 61	.	0. 51	0. 37	0. 72	.	2. 88	.	0. 58
Taurine	0. 04	0. 42	.	.	0. 03	.	.	.	.	.	.	.	0. 02	0. 08	0. 06	0. 49
Threonine	.	0. 93	.	0. 85	.	0. 62	.	0. 72	.	0. 39	.	0. 30	.	0. 58	.	0. 27
Tryptophane	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
Tyrosine	0. 03	0. 13	0. 04	0. 21	0. 04	0. 09	0. 08	0. 19	0. 06	0. 36	0. 17	0. 10	M	S	M	S
Valine	0. 14	1. 11	0. 13	1. 64	0. 20	1. 33	0. 15	1. 23	0. 16	0. 56	0. 33	0. 65	0. 06	*1. 82*	0. 05	*1. 24*
"X-spot"	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
'High? "Peptide" Str.'	+	.	+	.	+	.	.	.	.	.	.	.	+	.	+	.
Total	1. 43+	*14. 6*	1. 38+	*14. 6*	1. 22+	*9. 7*	1. 19+	*12. 0*	1. 09+	*9. 0*	[2] *4. 0*	9. 2+	0. 95+	*18. 5*	0. 58+	*13. 1*
NPN mg. per 100 ml	42	------	------	------	------	------	------	------	33	------	------	------	41	------	37	------
Protein of juice [2] (gm. %)	0. 18	------	------	------	------	------	------	------	0. 25	------	------	------	0. 13	------	0. 13	------
pH of juice [2]	4	------	------	------	5	------	------	------	8	------	------	------	3	------	2	------

Gastric juice specimen	#23		#20		#19		#16		Average values and limits	
Sample analyzed	Alcohol filtrate	Alcohol filtrate hydrolyzed	Alcohol filtrate	Alcohol filtrate hydrolyzed	Alcohol filtrate	Alcohol filtrate hydrolyzed	Alcohol filtrate	Alcohol filtrate hydrolyzed	Alcohol filtrate	Alcohol filtrate hydrolyzed
Alanine	0. 05	*1. 71*	0. 01	*1. 96*	0. 07	*0. 95*	0. 05	*3. 01*	0.07(0.01–0.13)	*1.53*(0.53–3.01)
γ-aminobutyric acid	0. 03	0. 15	.	*0. 33*	0. 05	0. 02	.	*0. 38*	0.13(N.D.–0.35)	*0.19*(0.02–0.38)
Arginine	0. 07	0. 11	.	0. 32	.	.	.	0. 49	(N.D.–0.10)	0.48(N.D.–1.16)
Aspartic acid	0. 01	*1. 34*	0. 03	0. 46	0. 08	*1. 21*	0. 09	*4. 23*	0.04(0.01–0.09)	*1. 47*(0.43–*4. 23*)
Cysteic acid (from cystine)	W	W	?	.	W	W	.	W	W(N.D.–W)	W(N.D.–W)
Glutamic acid	.	2. 36	0. 03	0. 47	0. 02	*0. 26*	0. 03	*1. 32*	0.03(N.D.–0.05)	*1. 37*(0.26–2.36)
Glutamine	0. 04	.	.	.	0. 11	.	0. 03	.	0.07(N.D.–0.12)	.
Glutathione	.	.	.	.	.	.	.	.	N.D.–W	.
Glutathione oxidized	.	.	.	.	W	.	.	.	W(N.D.–W)	.
Glycine	0. 05	*1. 65*	0. 13	*1. 41*	0. 04	*2. 49*	0. 02	*3. 21*	0.06(0.02–0.13)	*2. 16*(*1.41–3.21*)
Histidine	W	.	?	.	?	.	?	.	N.D.–W	.
Leucines	0. 36	*4. 02*	0. 16	*1. 57*	0. 23	*1. 56*	0. 28	*2. 68*	0.32(0.16–0.45)	*2.49*(1.02–*4.02*)
Lysine	.	0. 39	0. 07	*0. 58*	0. 04	0. 04	.	*0. 45*	0.07(N.D.–0.11)	*0.36*(0.04–*0.58*)
Methionine sulfone	.	.	?	.	.	.	.	.	.(.–?)	.
Phenylalanine	.	.	.	S	.	W	.	S	W(N.D.–W)	S(N.D.–S)
Proline	.	S	.	S	W	W	.	S	N.D.–W	S(N.D.–S)
Serine	.	0. 98	.	.	.	.	.	.	.	1.06(N.D.–2.88)
Taurine	.	.	0. 02	0. 18	.	.	.	.	0.04(N.D.–0.06)	0.29(N.D.–0.49)
Threonine	.	0. 47	0. 08	0. 03	.	0. 48	.	.	N.D.–0.08	0.47(N.D.–0.93)
Tryptophane	.	.	W	.	.	.	.	.	N.D.–W	.
Tyrosine	.	S	W	S	W	M	W	S	0.04(N.D.–0.06) M	0.19(0.09–0.36)S
Valine	0. 09	*1. 60*	0. 04	*1. 96*	0. 10	*0. 79*	0. 10	*3. 04*	0.10(0.04–0.20)	*1.49*(0.56–*3.04*)
"X-spot"	W	.	.	.	.	.	.	.	N.D.–W	.
'High? "Peptide" Str.'	.	.	+	.	+	.	+	.	+(N.D.–+)	.
Total	0. 70+	*14. 8*	0. 57+	*9. 3*	0. 74+	*7. 8*	0. 60+	*18. 8*	0.87+(0.57–1.43)	*12.8*(7.8–18.8)
NPN mg. per 100 ml	23		51		22				36(22–51)	
Protein of juice [2] (gm. %)	0. 09		0. 24		0. 12				0.16(0.09–0.25)	
pH of juice [2]	2		2		3		4			

[1] See Methods. An italicized value signifies that the "factor" for the amount of gastric juice chromatographed was greater than 150. The dots indicate that the amino acid was not detectable in the amount of gastric juice chromatographed. The "?" marks for cysteic acid (from cystine) and for methionine sulfone (from methionine) signify that no separate chromatograms were run with hydrogen peroxide and ammonium molybdate. The "?" marks on histidine signify no separate chromatograms were run for treatment with Pauly reagent. "W", "M", and "S" signify weak, medium, and strong color intensities of spots, respectively. A "+" sign indicates that a "High streak" was detectable. The averages on the table have been recorded in the following manner. With two or more detectable spots for a given amino acid in a series (as for the alcohol filtrates) the average of the two or more estimated concentrations of α-amino nitrogen has been recorded and the limits of the findings expressed as ("x–x") or ("N.D.–x") ("N.D." signifying that the amino acid was not always detectable). When a spot for an amino acid has occurred only once in a series, no average is recorded, but the limits of "N.D.–x" are shown. The averages of the totals were calculated from the total of each individual specimen analyzed. For the benefit of the reader the data have been tabulated in reverse order of the dates of study. In the more recent studies both alcohol filtrates and dialysates were chromatographed. Further, the authors believe their skills of qualitative identification and quantitative estimation became more accurate as the study progressed.

[2] The protein and pH values were measured on gastric juice filtered through gauze and paper (see Methods).

[3] The authors have no explanation for the unusually high amino-acid values for this dialysate; possibly amino acids were formed during the 24-hr. period of dialysis.



TEXT-FIGURE 2.—Graphic representation of the chromatograms of the amino acids before and after hydrolysis of dialysates of typical specimens of gastric juice and plasma from fasting normal individuals.

The areas of the spots are in direct proportion to the concentrations of the α–NH_2N as estimated from the "factors" gained from the actual chromatograms. Because the intensity of the spots is not depicted here, the sizes are not in the same relation as on the actual chromatograms (see Methods).

The solid spots represent the concentrations of the α–NH_2N of the free amino acids, the circles represent the concentrations of the α–NH_2N of the amino acids after acid hydrolysis of the dialysates.

"W" and "M" for ϕ-alanine, proline, and cysteic acid signify weak and medium spots, respectively. A "ν" signifies the amount was the same before and after hydrolysis. "d" signifies the spot disappeared on hydrolysis. In gastric juice #38 proline was detectable only after hydrolysis.

The numbers on the figure identify the amino acids as follows: 1. aspartic acid, 2. cysteic acid, 3. glutamic acid, 4. serine, 5. lysine, 6. glycine, 7. arginine, 8. threonine, 9. glutamine, 10. alanine, 11. tyrosine, 12. valine, 13. γ-aminobutyric acid, 14. leucines, 15. ϕ-alanine, 16. proline.

glycine, and proline in the gastric juice appear to be much less than in the plasma, the concentrations of the leucines and aspartic acid appear to be approximately the same in both fluids, and glutamic acid, γ-aminobutyric acid, taurine, and tyrosine were detected only, more frequently, or, in greater concentrations in the gastric juice.

The nonprotein-nitrogen values obtained on aliquots of the alcohol filtrates of the gastric juices used for chromatographic studies varied from 22 to 51 mg. per 100 ml. (table 2). The concentrations of the free and combined amino acids detectable on the chromatograms bore no relationship to the total nonprotein-nitrogen values of the alcohol filtrates and dialysates. The total protein values of the gastric-juice specimens varied from 0.09 to 0.25 gm. percent (table 2). The pH values of the juices varied roughly (*i. e.*, measured by test paper) from less than 2 to 8.

TABLE 3.—*Comparison of concentrations of free and combined amino acids in gastric juices of fasting normal individuals with concentrations of free and combined amino acids obtained 1 hour after an alcohol test meal in the same subjects, detectable on, and estimated from, chromatograms (Amino-acid values are expressed in mg. per 100 ml. of α-aminonitrogen)*

Gastric juice specimen	#23A		#23B		#25A		#25B	
When drawn	Fasting		1 hour after alcohol meal		Fasting		1 hour after alcohol meal	
Sample analyzed	Alcohol filtrate	Alcohol filtrate hydrolyzed	Alcohol filtrate	Alcohol filtrate hydrolyzed	Alcohol filtrate	Alcohol filtrate hydrolyzed	Alcohol filtrate	Alcohol filtrate hydrolyzed
Alanine	0.05	*1.71*	0.02	0.49	0.04	*1.09*	0.04	0.22
γ-aminobutyric acid	0.03	0.15	0.05	0.07	0.11	0.13	0.05	0.03
Arginine	0.07	0.11	.	0.26	.	.	.	0.22
Aspartic acid	0.01	*1.34*	0.01	*0.87*	0.01	*1.78*	0.01	0.45
Cysteic acid (from cystine)	W	W	.	W	.	.	?	.
Glutamic acid	.	*2.36*	.	*1.09*	.	1.82	0.07	0.63
Glutamine	0.04	.	0.04	.	0.04	.	.	.
Glutathione	.	.	.	.	.	.	M	.
Glutathione oxidized	.	.	.	.	W	.	W	.
Glycine	0.05	*1.65*	0.02	0.74	0.03	*1.74*	0.05	0.81
Histidine	W	.	W	.	?	.	W	.
Leucines	0.36	*4.02*	0.05	1.10	0.24	*2.54*	0.35	0.20
Lysine	.	0.39	.	.	.	0.22	.	.
Methionine sulfone	.	.	.	.	.	.	?	.
Phenylalanine	.	.	.	.	W	W	W	.
Proline	.	S	.	S	.	S	.	S
Serine	.	0.98	.	0.95	.	0.58	.	0.30
Taurine	.	.	.	.	0.06	0.49	.	.
Threonine	.	0.47	.	0.27	.	0.27	.	.
Tryptophane	.	.	.	.	.	.	.	.
Tyrosine	.	S	.	0.08	M	S	M	S
Valine	0.09	*1.60*	0.01	0.43	0.05	*1.24*	0.05	0.26
"X-spot"	W	.	.	.	.	.	.	.
'High ?"Peptide" Streak'	.	.	.	.	+	.	.	.
Total NPN mg. per 100 ml	0.70+ 23	*14.8*	0.20+ 27	*6.35*	0.58+ 37	*13.1*	0.62+ 20	3.12+
Protein gm. percent	0.09	------	0.11	------	0.13	------	0.10	------
pH of juice	2	------	2	------	2	------	7	------

DISCUSSION

Chromatographic studies as employed here fail to reveal the presence of many amino acids that exist in body fluids in amounts that are detectable by other methods of analysis. Thus, although there have been identified and quantitatively estimated, by chemical and microbiological methods, some 17 or more amino acids present in human plasma (table 1), our chromatograms on each of 2 plasma specimens (125 μl. amounts used) revealed the presence of only 9 spots for amino acids. Dent (*12*) has reported detection of a similar number of spots on chromatograms of human serum or plasma (250 μl. amounts used). The amino acids shown by chemical and microbiological methods to be in the greatest concentrations in plasma are those which were detectable on our chromatograms (table 1); it is also notable that most of the amino acids detected on the chromatograms are those which, relative to the others, give a stronger ninhydrin color reaction for a given amount of substance (*4*) (see Methods).

The chromatographic studies of the present report describe the identity of the "detectable" individual amino acids of the gastric juice of normal subjects (table 2) and also afford estimations of their concentrations. From the data gained by the same methods of analyses [14] on two normal human plasma specimens (table 1), it would appear that when an amino acid was detectable its concentration could be estimated with an informative degree of accuracy. It also appears that with the chromatographic methods utilized roughly two-thirds of the total α-amino-acid nitrogen of plasma, and presumably of gastric juice, was detectable in the amounts of fluid spotted onto the paper.

The concentrations of the individual amino acids of normal gastric juice differ markedly from those of plasma (table 1, text-fig.2) and of saliva (*24*). In gastric juice some two-thirds of the total detectable free α-NH_2N is comprised of the leucines plus valine and γ-aminobutyric acid; in contrast, in plasma approximately one-half of the detectable α-NH_2N is comprised of glutamine plus the leucines, and in saliva some two-thirds is comprised of glycine plus γ-aminobutyric acid. The average total detectable free α-NH_2N of gastric juice appears to be only approximately one-third that of plasma (tables 1, 2, text-fig. 2) and, in contrast, some four times that of

[14] It is worthy of note that we have detected glutamic acid on the chromatograms of only 3 alcohol filtrates of 13 specimens of normal human plasma studied and, that, in these 3 the average estimated amount was only 0.02 mg. per 100 ml. of α-NH_2N, contrasted with 0.32 (*18*) and 0.10 (*19*) mg. per 100 ml. reported from studies by microbiological and chemical methods. It is of interest that Krebs *et al.* (*18*) noted that the concentrations of glutamic acid plus glutamine in plasma and tissues showed much less variation than did the concentrations of the 2 components separately. These authors raised the question whether glutamic acid is formed from glutamine by the action of glutaminase in animal tissues after death, even in material treated with liquid air. The extreme ease with which glutamine undergoes acid hydrolysis is well known. Our chromatographic findings suggest that the validity of the many published results for the glutamic acid and glutamine concentrations of plasma and tissues by other methods is open to question presently.

It has been cited (*17*) that the concentration of proline constitutes only a negligible portion of the total amino acids in the plasma. Proline was almost always detectable on our chromatograms of plasma; it is very roughly estimated that proline is present in plasma on the order of magnitude equivalent to a concentration of 0.02 mg. of α-NH_2N per 100 ml.

The chromatographic method of Work (*11*) revealed the presence of l-leucine and isoleucine in approximately equal amounts in two plasma alcohol filtrate specimens studied; this proportionality accords with that found by microbiological methods (*16*).

saliva (*24*). Because of the above-stated differences, a chromatogram of a specimen of normal gastric juice is easily distinguishable from that of a normal plasma or saliva.

That there was found considerable variation in the amino-acid concentrations of different specimens of gastric "contents" from normal fasting individuals (table 2) was to be expected. The gastric juice itself is a composite of secretions from at least three different types of cells in the gastric mucosa. In addition, the fasting gastric juice is "contaminated" with varying small amounts of saliva (which is variable in total amino-acid concentration and differs from the amino-acid composition of gastric juice), and at times with small amounts of regurgitations from the duodenum. There is evidence that the parietal-cell secretion is, essentially, if not entirely, nitrogen free (*25*, *26*).

It is of interst that γ-aminobutyric acid, which is often not detectable in the normal human plasma of adults [table 1; (*27*)] and is not known to be a constituent of any protein (*27*), has been found to constitute a very appreciable percentage of the total amino nitrogen of certain secretions that we have studied, namely, gastric juice [Results, (*1*)], saliva (*24*), and cerebrospinal fluid.[15] It is pertinent in this connection that Crumpler *et al.* (*27*) found the γ-aminobutyric-acid concentration of fetal plasma to be disproportionately greater in relation to maternal plasma than were the other amino acids. The mechanism of "concentration" of amino acids by the placenta is not known (*27*).

That the total α-amino nitrogen of gastric-juice alcohol filtrates and dialysates increased greatly (some 10 times) in concentration on hydrolysis indicates the presence of large amounts of amino acids in peptide linkage (text-fig. 2, table 2). The similar findings on hydrolysis of alcohol filtrates and dialysates of the same juices indicate that the alcohol filtrates were free, largely, of protein. The large increase of amino acids after hydrolysis may be due to the presence of many peptides of low-molecular weight or a few with high-molecular weight.

The weak unidentified purple spot (termed "streak") (text-fig. 1) that occurred with rather great frequency on the chromatograms of the gastric juice (table 2) and that was not present after hydrolysis, would appear from preliminary elution experiments to be due to a single polypeptide or to several overlapping peptides constituted of many amino acids. We assume, until it is otherwise proved, that this "high streak" was not due to the presence of protein in the alcohol filtrates or dialysates. It is notable that this streak was not seen on the chromatograms of the alcohol filtrates of any of 12 plasma specimens or any of 8 saliva specimens (*24*) that we have studied. Possibly the weak unidentified purple spot (termed "X–Spot") (text-fig. 1), which we detected on the chromatograms of 1 normal gastric juice (#23) (table 2) at R_f values of approximately 0.30 and 0.09 in phenol and "collidine" respectively, and which disappeared on hydrolysis, corresponds to "Spot W" described by Roberts and Frankel

[15] A "good" spot for γ-aminobutyric acid was seen on the one chromatogram of cerebrospinal fluid that we have run.

(*28*), who have detected this spot in extracts of tissues, *etc.*, and suspect it to be, on the basis of preliminary isolation experiments, a polypeptide that contains at least 9 or 10 amino acids. The positive identification of the peptides in gastric juice was beyond the scope of this paper.

The only published data on the total amino-acid concentration of normal gastric juice that we have been able to find are those of Martin (*29*, *30*), gained through analyses by the unmodified, nonspecific, Folin (*31*) method.

The nonprotein-nitrogen and total-nitrogen values found in this study of normal gastric juices from fasting individuals are within the range of values reported by others (*25*, *29*, *30*).

Many different technics for estimating quantitatively the individual amino acids after their separation by paper chromatography have been described recently (*32*, *33*); these technics have largely been concerned with one-dimensional paper chromatograms or with column fractionators. It is to be noted that, providing there is no overlapping of spots of different amino acids, quantitation from one-dimensional paper chromatograms can be made by relatively simple "spot-dilution" methods (*32*, *34*, *35*). As explained above, however, one-dimensional paper-chromatographic methods are very unsatisfactory for the separation of the many amino acids present in body fluids. Woiwod (*36*, *37*) has demonstrated that losses of amino-nitrogen occur on both one- and two-dimensional paper chromatograms, the losses being greater for the two-dimensional method, and progressively greater with increasing distance traveled by a given amino acid. Adsorption of the amino acids, a failure of the acids to reach equilibrium with the solvent in the moving phase (*36*) and/or a reaction of amino acids and allied compounds with aldehydic groups derived from modified or short-chain celluloses present in the filter paper (*37*) have been offered as reasons for these losses. It is to be noted that such losses should not affect materially quantitative estimations by the method utilized in our work, since they would be expected to be equivalent for the knowns and unknowns that were chromatographed in exactly the same manner.

The simple empirical method of quantitation that we have utilized, *i. e.*, measuring spot intensity against a color chart and spot size, and combining these two measurements to give a "factor" (size $\times$ intensity) which, when compared with the "factors" attained by the same methods for known amounts of amino acids, has allowed estimations of an informative degree of accuracy of the concentrations of many of the amino acids in gastric juice. Although Fisher, Parsons, and Morrison (*38*) have found that on one-dimensional strips the area of the spot is proportional to the logarithm of the amount of amino acid, our results agree with the observations of Work (*11*)—that this correlation of spot size alone with the amount of amino acid does not hold well for two-dimensional chromatograms. Similarly, our studies on standards demonstrated that measurement of spot intensity alone usually does not afford a reliable means of quantitation on two-dimensional chromatograms.

Methods for quantitative estimations which are somewhat similar to that employed here have been utilized by others (*2, 11, 12, 39, 40*). Although the spot size was not taken into consideration in their studies, Dent and Schilling (*2*) have utilized a color-intensity chart to show the relative ninhydrin coloration in two-dimensional chromatograms of amino-acid spots from dog plasma. Work (*11*) has estimated the amount of amino acids present in unknown mixtures by comparing both the color intensity of spots against an arbitrary scale and the size of the spots with those given by known amounts of pure amino acids run under identical conditions on two-dimensional chromatograms. Ames and Risley (*39*) estimated the color intensity on one-dimensional strips from "1" to "5" (where "1" represented the faintest band observable and "5" represented an intensely colored band) and also measured the width of the band, and thereby obtained a "dw" value by multiplying the intensity by the width in centimeters. Block (*40*) has quantitatively estimated the amino acids on paper chromatograms by multiplying the greatest color density, measured with an electronic densitometer, by the area of the spot, and relating these values to those of standards treated in the same manner. It is to be remembered that methods that utilize a color chart for estimation of intensity of standards and unknowns, even when combined with spot size, as we have done, have the disadvantage of lack of control over variations in different runs, which control at least is gained by comparing multiple dilutions of unknowns with multiple dilutions of standards all run at the same time, in the same tank, and under identical conditions (*34, 40, 41*); the latter could not be accomplished in the simple chromatographic apparatus utilized in this study.

It did not appear feasible to us for the immediate purposes of the present study to employ starch-column fraction cutters (*32, 33*), or one of the more time-consuming procedures for quantitation from two-dimensional chromatograms that have been suggested (*32, 33*), although some refinement seems entirely desirable for further, more accurate, quantitations. The use of the electrolytic "desalting" procedure (*42*) would enhance the ease of identification of the amino acids by paper chromatographic methods, and allow the employment of much larger quantities of body fluids in the chromatographic studies, thereby increasing the number of detectable ninhydrin-reacting substances, and the accuracy of quantitations. The employment for chromatograms of ultrafiltrates (*2, 27*) or of dialysates (table 2) of gastric juice in place of alcohol filtrates, is probably also desirable for future study of the total peptide α-NH_2N concentrations. It would seem important also, in further studies, that the estimations of the total free and peptide amino acids of gastric juice as detectable and measured by chromatography be compared with the values obtained by the Van Slyke gasometric method of measurement (*43, 44*), and that chromatographically estimated values for the individual amino acids be compared with those obtained by refined chemical or microbiological methods (table 1).

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SUMMARY AND CONCLUSIONS

Information on the identity and concentrations of the individual amino acids in the gastric juice of normal individuals has been gained by a technic of two-dimensional paper-partition chromatography. Nonprotein nitrogen, protein, and acid concentrations of the gastric juices have also been studied.

The methods utilized for the detection and quantitative estimations of the amino acids and other ninhydrin-reacting substances have been outlined in detail. The methods afforded accurate qualitative information concerning most of the detectable ninhydrin-reacting substances in the body fluids studied; the quantitative estimations gave results of an informative degree of accuracy. The methods employed in this study are discussed in relation to methods recently utilized by others. The pitfalls of interpretations are outlined.

The chromatograms of the gastric juice of fasting normal individuals always revealed the presence of alanine, aspartic acid, glycine, the leucines, and valine; in addition, γ-aminobutyric acid, glutamic acid, glutamine, tyrosine, and an unidentified "high streak" (which was not present on hydrolyzed filtrates) were usually detectable, and weak spots for certain other amino acids were less frequently detected.

It has been estimated that the average total free detectable α-NH_2N concentration of gastric juice is approximately 0.9+ mg. per 100 ml.; *i. e.*, approximately one-third that of plasma and four times that of saliva. Certain data indicate that roughly two-thirds of the α-NH_2N of body fluids is "detectable" by the chromatographic method utilized. The "pattern" of the free amino acids of plasma differs greatly from the patterns for plasma and saliva. Approximately two-thirds of the total detectable free α-NH_2N of gastric juice is comprised of the leucines, valine, and γ-aminobutyric acid.

The estimated concentration of the detectable α-NH_2N from "peptides" in normal gastric juice averaged approximately *12* mg. per 100 ml., as compared with the value of 0.9+ mg. per 100 ml. for the free amino acids. The nature of the "peptides" in gastric juice was not clarified in these studies; the little information gained has been reported. The detectable α-NH_2N concentration in "peptide" linkage in gastric juice is markedly greater than in plasma and exceeds that of saliva by some eight times.

The nonprotein nitrogen and protein values found in our studies of normal gastric juice agree with those reported by others.

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PAPER PARTITION CHROMATOGRAPHY OF AMINO ACIDS OF GASTRIC JUICE OF PATIENTS WITH GASTRIC LESIONS.[1] [2]

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INTRODUCTION

An investigation by two-dimensional paper partition chromatography of the amino acids and peptides of the gastric juice of patients with peptic and duodenal ulcers, and carcinoma of the stomach has been made by the same methods utilized in studies of the gastric juice of normal individuals (*1*). Findings for the total-protein concentrations, the nonprotein-nitrogen concentrations, and the acidities of the gastric juices are also reported. The results of these studies in patients with gastric lesions, herein reported, are discussed below as related to those found for normal individuals (*1*). The feasibility of the methods encouraged further investigation to discover whether knowledge of the amino-acid concentration of the gastric juice might afford aid in the differential diagnosis of a gastric lesion.

Since the pathology of gastric cancer indicates that there must be at least partial digestion of the tumor substance, it seemed logical to expect a higher amino-acid content of the gastric secretion in gastric-cancer cases. This conclusion is forced by 1) the small size of gastric cancers in relation to the mitotic rate, 2) the almost constant ulceration of gastric cancers, 3) the absence of extensive foci of necrosis; all of which indicate lysis of the tumor by proteolytic enzymes.

MATERIAL

Gastric-juice specimens were obtained from patients in the New England Deaconess Hospital.[4] The specimens were aspirated, using a Levin-tube aspirator, after the subjects had fasted overnight.

Two-dimensional chromatograms were run on the fasting gastric juice from 21 subjects, comprising 6 patients with carcinoma of the stomach, 3 with gastric, and 4 with duodenal ulcers, 1 with "gastric ulcer, ? benign," 1 with carcinoma, probably of linitis plastica type, 1 who had had a partial gastrectomy 9 days before study, 1 who had had a gastroenterostomy 13 years previously, and 4 other patients whose gastric juice was obviously

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grossly contaminated with bile, blood, or food. The findings in the latter 7 patients are not included in the tabulated data but receive comment in the text. In addition, chromatographic studies were made on gastric juice obtained after an alcohol meal (*1*) in 2 of the patients with carcinoma of the stomach.

METHODS

The chromatographic and chemical methods utilized in this investigation have been described by the authors in detail elsewhere (*1*). The chromatograms were run in phenol followed by collidine-lutidine. The free amino acids of the gastric juice of the patients with ulcers were chromatographed from amounts of alcohol filtrates and dialysates equivalent to 125μl. of juice, and those of the patients with carcinoma from amounts equivalent to from 31.3μl. to 125μl. of juice. The amino acids of the hydrolyzed alcohol filtrates and dialysates were chromatographed from amounts of hydrolysate equivalent to from 15.6μl. to 62.5μl. of juice.

It might have been preferable if, in the patients with gastric carcinoma, amounts of filtrate equivalent to very small amounts of gastric juices were employed, as the range of values of amino acids would be thus more nearly parallel to those in normal cases. A number of the gastric juices from cases of carcinoma showed over one-hundred-fold the amount of amino acids as in normal persons. However, in most instances, the diagnosis of the underlying lesion was not known at the time of investigation of the gastric juice.

The manner and significance of the quantitative estimations of the α-NH_2N concentrations of the individual amino acids from chromatograms as employed here have been discussed elsewhere (*1*). The usage of the terms "detectable," "amino acid," "α-NH_2N" and "peptide" in this text also have been explained (*1*). In two of the patients with peptic ulcer and two with carcinoma of the stomach chromatographic studies were made prior to the time we utilized the color chart for estimation of intensity of spots (*1*). In these instances and in those of the studies of two fasting gastric-juice specimens from normal subjects the intensities of the spots were estimated roughly, in percentage of the intensity of an alanine standard chromatographed at the same time, and the size of the spot was measured. The estimated (against alanine) intensity was multiplied by the size of the spot and the results thus obtained were finally expressed by grading in terms of +'s, from $<1+$ to $>>>10+$.

RESULTS

The chromatograms of the gastric juices of the fasting patients with gastric and duodenal ulcers (tables 1 and 2) were so similar in most instances to those for normal individuals (*1*) that they deserve no further comment. The chromatogram of the gastric juice of one patient with a gastric ulcer (Specimen #33,[5] table 1) revealed the concentrations of the amino acids to be approximately three times as great as were found on the average for normal individuals or for the other patients with ulcers. In the one case of this study (Specimen #12, table 2), in which the differential diagnosis between gastric ulcer and carcinoma was not established, the chromatographic study revealed a very much higher total amino-acid concentration of the gastric juice than was observed for normal individuals or for any of the patients with ulcers. This patient was a woman, aged 81 years. She was found to have an ulcer of the lesser curvature, and to be achlorhydric. On X-ray examination 6 weeks subsequent to the time of our chromatographic study the ulcer was still observed. The diagnosis of cancer seems probably correct.

In each of the six patients with carcinoma of the stomach the chromatographic studies revealed very much higher (averaging some 15 times higher) concentrations of detectable amino acids in the gastric juice than found in normal individuals (tables 2 and 3). Not only were the spots for most of the amino acids on the chromatograms in these patients much more intense than those observed for normal subjects, but usually more spots were seen on a given chromatogram.

In these patients with gastric carcinoma the identity of the detectable amino acids and other ninhydrin-reacting substances was for the most part the same as were detectable in smaller amounts and/or with less frequency on the chromatograms of normal individuals. Despite the large increases in the concentrations of the individual amino acids most frequently detected, their average relative proportionalities in a given specimen were essentially the same in the patients with carcinoma of the stomach as in normal individuals. In the patients with carcinoma, alanine, the leucines, and valine were always markedly increased above the normal. Methionine sulfone, which was not detectable on any of the chromatograms of the gastric juice of normal individuals or patients with ulcers, appeared on the chromatograms, after treatment with H_2O_2 and ammonium molybdate, of the gastric-juice filtrates of three patients with carcinoma of the stomach (table 3).

[5] It may be worthy of note that difficulty was encountered, after the Levin tube had been inserted, in obtaining this gastric-juice specimen, and that the pH of the juice was 7, suggesting that the specimen may have been contaminated by duodenal regurgitation.

TABLE 1.—*Concentrations of free and combined amino acids in gastric juices of fasting patients with gastric and duodenal ulcers, detectable on, and estimated from, chromatograms.*[1] *(Amino-acid values are expressed in mg. per 100 ml. of α-amino nitrogen)*

Site of ulcer	Duodenum				Duodenum	Duodenum	Stomach
Gastric-juice specimen	#37				#35	#34	#33
Sample analyzed	Alcohol filtrate	Alcohol filtrate hydrolyzed	Dialysate	Dialysate hydrolyzed	Alcohol filtrate	Alcohol filtrate	Alcohol filtrate
Number of samples							
Alanine	0. 08	0. 77	0. 08	0. 73	0. 18	0. 09	0. 23
γ-aminobutyric acid	0. 15	0. 23	0. 08	0. 24	0. 21	0. 09	0. 15
Arginine	.	0. 56	.	0. 52	.	.	0. 38
Aspartic acid	0. 02	0. 67	0. 02	0. 85	0. 03	0. 04	0. 07
Cysteic acid (from cystine)	W	W	W	W	W	W	.
Glutamic acid	0. 02	0. 52	0. 02	0. 47	0. 02	0. 02	0. 06
Glutamine	0. 11	.	0. 09	.	0. 05	0. 17	0. 21
Glutathione	.	.	.	.	W	W	.
Glutathione oxidized	W	.	W	.	W	W	W
Glycine	0. 12	1. 86	0. 17	1. 59	0. 13	0. 12	0. 38
Leucines	0. 26	1. 70	0. 21	1. 84	0. 27	0. 23	0. 65
Lysine	0. 03	0. 27	0. 04	0. 28	0. 09	.	0. 05
Methionine sulfone	?	.	?	.	.	.	.
Phenylalanine	.	.	.	.	.	.	W
Proline	.	S	.	S	.	.	.
Serine	.	0. 23	.	0. 18	.	.	.
Taurine	.	0. 15	.	0. 08	0. 06	0. 08	.
Threonine	.	.	.	.	.	.	.
Tyrosine	.	0. 18	.	0. 11	W	.	M
Valine	0. 06	0. 61	0. 10	0. 53	0. 15	0. 14	0. 27
"X-Spot"	.	.	.	.	.	.	.
"High Streak"	.	.	.	.	+	.	.
Total	0. 85+	7. 75+	0. 81+	7. 32+	1. 19+	0. 98+	2. 45
NPN mg. per 100 ml	26		24		71	48	39
Protein gm. percent [2]	0. 09				0. 20	0. 18	0. 36
pH of juice [2]	2				2	3	7

Site of ulcer	Stomach		Duodenum					
Gastric-juice specimen	#30		#28		Average	Average	Average of normals [1]	
Sample analyzed	Alcohol filtrate	Alcohol filtrate hydrolyzed	Alcohol filtrate	Alcohol filtrate hydrolyzed	Alcohol filtrate	Alcohol filtrate hydrolyzed	Alcohol filtrate	Alcohol filtrate hydrolyzed
Number of samples					6	3	9	9
Alanine	0. 10	*1. 01*	0. 08	*1. 26*	0. 13	*1. 01*	0. 07	*1. 53*
γ-aminobutyric acid	0. 42	0. 61	0. 22	0. 29	0. 21	0. 38	0. 13	*0. 19*
Arginine	.	0. 40	.	1. 22	N. D.–0. 38	0. 73	N.D.–0. 10	0. 48
Aspartic acid	0. 06	*1. 56*	0. 05	*1. 46*	0. 04	*1. 23*	0. 04	*1. 47*
Cysteic acid (from cystine)	.	W	?	W	W	W	W	W
Glutamic acid	0. 04	0. 52	0. 04	*0. 96*	0. 03	*0. 33*	0. 03	*1. 37*
Glutamine	0. 12	.	0. 05	.	0. 12	.	0. 07	.
Glutathione	.	.	.	.	W	.	N.D.–W	.
Glutathione oxidized	W	.	.	.	W	.	W	.
Glycine	0. 12	0. 72	0. 04	1. 14	0. 15	1. 24	0. 06	*2. 16*
Leucines	0. 16	*2. 57*	0. 08	*3. 00*	0. 28	*2. 42*	0. 32	*2. 49*
Lysine	0. 10	0. 49	.	.	0. 07	0. 38	0. 07	*0. 36*
Methionine sulfone	.	.	?	.	.	.	.	.
Phenylalanine	.	.	.	.	N.D.–W	.	W	S
Proline	.	M	.	S	.	S	N.D.–W	S
Serine	.	0. 18	.	0. 90	.	0. 44	.	*1. 06*
Taurine	0. 05	.	0. 05	.	0. 06	N.D.–0. 15	0. 04	0. 29
Threonine	.	1. 06	.	1. 44	.	1. 25	N.D.–0. 08	0. 47
Tyrosine	W	0. 25	.	0. 16	W	0. 20	0. 04 (M)	0. 19 (S)
Valine	0. 05	*1. 39*	0. 44	*1. 77*	0. 12	*1. 26*	0. 10	*1. 49*
"X-Spot"	W	.	.	.	N.D.–W	----------	N.D.–W	.
"High Streak"	+	.	+	.	+	----------	+	.
Total	1. 22+	*10. 8*	0. 65+	*13. 6*	1. 22+	*10. 7*	0. 87+	*12. 8*
NPN mg. per 100 ml	50	----------	0. 41	----------	46	----------	36	----------
Protein gm. percent [2]	0. 24	----------	0. 17	----------	0. 21	----------	0. 16	----------
pH of juice [2]	2	----------	2	----------	----------	----------	----------	----------

[1] See Methods. An italicized value signifies that the "factor" for the amount of gastric juice chromatographed was greater than 150. The dots indicate that the amino acid was not detectable in the amount of juice chromatographed. The "?" marks of cysteic acid (from cystine) and for methionine sulfone (from methionine) signify that no separate chromatograms were run with hydrogen peroxide and ammonium molybdate. "W", "M", and "S" signify weak, medium, and strong color intensities of spots, respectively. The averages on the table have been recorded in the following manner: With two or more detectable spots for a given amino acid in a series (as for the alcohol filtrates) the average of the two or more estimated concentrations of α-amino nitrogen has been recorded. When a spot for an amino acid has occurred only once in a series, no average is recorded, but the limits of "(N.D.-x)" are shown, "N.D." signifying that the amino acid was not detectable. The averages of the totals were calculated from the total of each individual specimen analyzed. The average values found for normal gastric juice (*1*) have been recorded for purposes of comparison. [2] N.B. The protein and pH values were measured on gastric juice filtered through gauze and S. & S. #604 paper.

TABLE 2.—*Comparison of chromatograms* [1] *of the amino acids in fasting gastric juices of normal individuals, patients with peptic ulcer and patients with carcinoma of the stomach* [2]

Diagnosis	Normal		Ulcer	?Ulcer ?Carcinoma	Carcinoma	
Gastric-juice specimen	#5	#6	#9	#12	#10	#11
Sample analyzed	Alcohol filtrate	Alcohol filtrate	Alcohol filtrate	Alcohol filtrate	Alcohol filtrate	Alcohol filtrate
Alanine	1+	1+	<1+	8+	>>10+	4+
γ-aminobutyric acid	1+	<1+	2+	<1+	>>10+	<1+
Aspartic acid	1+	<1+	<1+	<1+	1+	>10+
Cysteic acid (from cystine)	?	?	?	?	?	?
Glutamic acid	<1+	.	<1+	<1+	1+	<1+
Glutamine	<1+	.	<1+	<1+	<1+	.
Glutathione	.	<1+	.	<1+	.	.
Glycine	<1+	<1+	.	<1+	1+	4+
Leucines	1+	4+	2+	>>10+	>10+	>>>10+
Lysine	<1+	.	<1+	.	<1+	.
Methionine sulfone	?	?	?	?	?	?
Phenylalanine	.	.	.	.	W	W
Proline	.	.	W	.	M	.
Taurine	<1+	<1+	.	.	.	<1+
Threonine	<1+	.	.	.	.	.
Tryptophane	.	.	.	.	<1+	.
Tyrosine	.	.	W	M	M	M
Valine	<1+	<1+	<1+	3+	7+	4+
"High Streak"	+	+	+	.	.	+

[1] The size of the spot was multiplied by the estimated intensity in terms of an alanine standard and the results thus obtained were expressed in terms of +'s, graded from <1+ to >>>10+.
[2] See footnotes of table 1.

The chromatograms (table 4) of the juices after alcohol test meals, as well as those of the juices in the fasting state, revealed more spots and much stronger spots for the amino acids in the studies of two patients with cancer of the stomach than were observed in two normal subjects similarly studied (*1*). In one of these patients with gastric carcinoma the concentrations of the free amino acids were the same in the fasting gastric-juice specimen and in the specimen obtained 1 hour after the alcohol meal; in the other patient the free amino acid concentration after the alcohol meal was only approximately one-third that of the fasting specimen. Since studies were obtained after the alcohol meal in only these few subjects, the findings are not further discussed.

On the average, the concentrations of the amino acids in peptide form in the gastric juice of the patients with ulcers were found high in respect to the concentrations of free amino acids, and similar in kind and amount to those in the gastric juice of normal individuals (table 1).

TABLE 3.—*Concentrations of free and combined amino acids in gastric juices of fasting patients with carcinoma of the stomach, detectable on, and estimated from, chromatograms* [1] *(Amino-acid values are expressed in mg. per 100 ml. of α-amino nitrogen)*

Gastric-juice specimen	#27		#24	#22		#21		Average of carcinomas		Average of normals (1)	
Sample analyzed	Alcohol filtrate	Alcohol filtrate hydrolyzed	Alcohol filtrate	Alcohol filtrate	Alcohol filtrate hydrolyzed	Alcohol filtrate	Alcohol filtrate hydrolyzed	Alcohol filtrate	Alcohol filtrate hydrolyzed	Alcohol filtrate	Alcohol filtrate hydrolyzed
Number of samples								4	3	9	9
Alanine	*2. 17*	*2. 99*	*0. 66*	*1. 14*	*1. 16*	*2. 22*	*6. 28*	*1. 55*	*3. 48*	0. 07	*1. 53*
γ-aminobutyric acid	0. 44	0. 44	*1. 24*	0. 10	0. 07	0. 10	0. 23	*0. 47*	0. 25	0. 13	*0. 19*
Arginine	1. 83	1. 81	.	0. 08	0. 46	*1. 97*	*0. 74*	*1. 29*	*1. 00*	N.D.–0. 10	0. 48
Aspartic acid	0. 25	*1. 51*	0. 04	0. 26	*1. 06*	*0. 42*	*2. 32*	*0. 24*	*1. 63*	0. 04	*1. 47*
Citrulline and/or β-alanine	.	.	.	.	.	M	M	N.D.–M	N.D.–M	.	.
Cysteic acid (from cystine)	W	W	.	.	?	W	W	W	W	W	W
Glutamic acid	0. 30	*3. 24*	0. 11	0. 32	*2. 07*	0. 23	*1. 21*	0. 24	*2. 17*	0. 03	*1. 37*
Glutamine	0. 98	.	0. 38	0. 22	.	.	.	0. 53	.	0. 07	.
Glutathione	.	.	.	W	.	.	.	N.D.–W	.	N.D.–W	.
Glutathione oxidized	W	.	W	W	.	W	.	W	.	W	.
Glycine	0. 98	*3. 86*	0. 24	*2. 16*	*5. 25*	0. 58	*2. 58*	*0. 99*	*3. 90*	0. 06	*2. 16*
Histidine	?	?	?	W	?	W	?	W	?	N.D.–W	.
Leucines	*8. 47*	*12. 08*	*1. 43*	*2. 06*	*3. 02*	*6. 62*	*7. 82*	*4. 64*	*7. 64*	0. 32	*2. 49*
Lysine	1. 80	*1. 67*	0. 25	0. 16	0. 94	0. 16	0. 16	0. 59	*0. 92*	0. 07	*0. 36*
Methionine sulfone	.	.	W	.	.	W	W	W	N.D.–W	.	.
Methionine sulfoxide	.	.	.	W	W	.	.	N.D.–W	N.D.–W	.	.
Phenylalanine	.	.	S	.	.	S	S	S	N.D.–S	W	S
Proline	W	S	M	M	S	M	>S	M	S	N.D.–W	S
Serine	0. 67	0. 92	.	0. 12	0. 09	.	.	0. 38	0. 50	.	*1. 06*
Taurine	0. 15	0. 43	0. 18	.	.	0. 21	0. 18	0. 18	0. 30	0. 04	0. 29
Threonine	1. 07	0. 99	.	0. 08	0. 29	.	0. 32	0. 58	0. 53	N.D.–0. 08	0. 47
Tyrosine	0. 59	0. 95	S	S	S	S	S	0. 59	0. 95	0. 04(M)	0. 19(S)
Valine	*3. 74*	*3. 18*	*0. 62*	*0. 93*	*1. 09*	*1. 71*	*4. 43*	*1. 75*	*2. 90*	0. 10	*1. 49*
"X-Spot"	.	.	S	.	.	.	.	N.D.–S	.	N.D.–W	.
"High Streak"	.	.	+	.	.	.	.	N.D.–+	.	+	.
Total	*23. 4*	*34. 1*	*5. 15*	*7. 63*	*15. 5*	*14. 2*	*26. 3*	*12. 6*	*25. 3*	0. 87+	*12. 8*
NPN mg. per 100 ml	64	------	118	42	------	116	------	85	----------	36	----------
Protein gm. percent [2]	0. 59	------	0. 51	0. 29	------	0. 38	------	0. 44	----------	0. 16	----------
pH of juices [2]	7	------	4	7	------	5	------	----------	----------	----------	----------

[1] and [2]: See footnotes of table 1.

TABLE 4.—*Comparison of concentrations of free and combined amino acids in fasting gastric juice and juice withdrawn 1 hour after an alcohol test meal in patients with carcinoma of the stomach; detectable on, and estimated from, chromatogens.*[1] *(Amino-acid values are expressed in mg. per 100 ml. of α-amino nitrogen)*

Gastric-juice specimen	#22				#27			
When drawn	Fasting		One hour after alcohol meal		Fasting		One hour after alcohol meal	
Sample analyzed	Alcohol filtrate	Alcohol filtrate hydrolyzed	Alcohol filtrate	Alcohol filtrate hydrolyzed	Alcohol filtrate	Alcohol filtrate hydrolyzed	Alcohol filtrate	Alcohol filtrate hydrolyzed
Alanine	*1.14*	*1.16*	*1.14*	0.75	*2.17*	*2.99*	0.33	*3.36*
γ-aminobutyric acid	0.10	0.07	0.03	0.07	0.44	0.44	0.18	0.54
Arginine	0.08	0.46	0.06	0.50	1.83	1.81	0.87	*3.13*
Aspartic acid	0.26	*1.06*	0.07	0.41	0.25	*1.51*	0.14	*3.56*
Cysteic acid (from cystine).	.	?	.	W	W	W	?	W
Glutamic acid	0.32	*2.07*	0.14	*0.98*	0.30	*3.24*	0.25	*3.66*
Glutamine	0.22	.	0.19	.	0.98	.	0.21	.
Glutathione	W	.	M	.	.	.	.	.
Glutathione oxidized	W	.	W	.	W	.	.	.
Glycine	*2.16*	*5.25*	*1.51*	*4.35*	0.98	*3.86*	0.26	*5.71*
Histidine	W	.	W	.	?	?	?	.
Leucines	*2.06*	*3.02*	*2.66*	*2.86*	*8.47*	*12.08*	*3.09*	*10.20*
Lysine	0.16	0.94	0.30	.	1.80	*1.67*	0.08	0.72
Methionine sulfone	.	.	W	.	.	.	?	S
Methionine sulfoxide	W	W	M	W	.	.	.	.
Phenylalanine	.	.	.	.	.	.	.	.
Proline	M	S	M	S	W	S	W	>S
Serine	0.12	0.09	0.12	0.05	0.67	0.92	0.83	*3.20*
Taurine	.	.	.	.	0.15	0.43	0.05	0.19
Threonine	0.08	0.29	0.08	0.30	1.07	0.99	0.31	1.88
Tryptophane	.	.	.	.	.	.	.	.
Tyrosine	S	S	S	S	0.59	0.95	S	0.59
Valine	*0.93*	*1.09*	*0.96*	0.77	*3.74*	*3.18*	*0.55*	*3.86*
"X-Spot"	.	.	M	.	.	.	.	.
"High streak"	.	.	.	.	.	.	+	.
Total	*7.6*	*15.5*	*7.3*	*11.0*	*23.4*	*34.1*	*7.2*	*40.6*
NPN mg. per 100 ml	42	------	36	------	64	------	92	------
Protein gm. percent [2]	0.29	------	0.25	------	0.59	------	0.47	------
pH of juice [2]	7.5	------	7.0	------	7.0	------	6.0	------

[1] and [2]: See footnotes of table 1.

Although the concentrations of free amino acids, and of total amino acids after hydrolysis of alcohol filtrates, of the gastric juices of the patients with carcinoma of the stomach greatly exceeded those of normal individuals and patients with ulcers, the concentrations of amino acids in peptide form did not appear increased above normal in the three specimens studied (table 3).

The nonprotein-nitrogen concentration of the fasting gastric juice of the patients with gastric and duodenal ulcers averaged 46 (26–71) mg. per 100 ml., and that of the patients with carcinoma of the stomach 85 (42–118) mg. per 100 ml.; these values are to be compared with 36 (22–51) mg. per 100 ml. of nonprotein nitrogen in the gastric juice of the normal individuals studied [tables 1 and 3, and ref. (*1*)]. There became evident, on averaging, a tendency toward higher concentrations of free and total amino acid in the presence of higher concentrations of nonprotein nitrogen,

although a direct proportionality between the amino acid and nonprotein-nitrogen concentrations of individual specimens of gastric juice frequently was not found.

The average protein concentration of the fasting gastric-juice specimens from the patients with ulcers was 0.21 gm. per 100 ml., for the patients with gastric carcinoma 0.44 gm. per 100 ml., as compared with that for the normal individuals of 0.16 gm. per 100 ml. [tables 1 and 3, and ref. (*1*)].

In a patient (#7) who had had a gastroenterostomy for peptic ulcer 13 years previous to our study and who recently had had repair of a gastro-jejunocolic fistula, the chromatogram of the gastric juice was essentially "normal." In another patient (#8) who had had a gastroenterostomy for treatment of peptic ulcers of the pylorus 9 days previous to our studies the chromatogram of the gastric juice showed higher concentrations of amino acids than were found in normal subjects; in fact, the chromatogram was not unlike the "weaker" chromatograms of the juices of certain of the patients with gastric carcinoma (table 3). The chromatogram of the gastric juice of a patient (#14) with metastatic carcinoma of the omentum, who was observed at operation to have a typical linitis plastica, was entirely like that of those from normal individuals. This might well be expected in view of the virtually intact mucosa that characterizes linitis plastica.

Four juices were obviously contaminated with bile, blood, or food. The chromatograms of two gastric juices that were grossly contaminated with lower intestinal contents and that were aspirated from a patient with peptic ulcer (#18) and a patient with gastric carcinoma (#32) showed very high concentrations of a very great number of amino acids. In fact, there were more amino acids detectable and in higher concentrations in these specimens than in any of the "uncontaminated" juices studied, including the juices of patients with gastric carcinoma. In one patient with carcinoma of the stomach (#15) whose juice was contaminated with blood ("coffee-grounds material"), the chromatogram of the unhydrolyzed gastric-juice filtrate was not unlike the "stronger" chromatograms of other patients with carcinoma of the stomach. (The nonprotein nitrogen and protein concentrations were exceedingly high in this juice, being 212 mg. per 100 ml. and 1.09 gm. percent, respectively.) In a patient with duodenal ulcer and obstruction, whose juice was contaminated with food (?crackers), (#17), the chromatogram of the unhydrolyzed gastric-juice filtrate was not unlike the chromatograms of normal subjects; however, the chromatogram of the hydrolysate of the alcohol filtrate revealed more and stronger spots than the hydrolysates of juices of normal subjects or of other patients with duodenal ulcers.

DISCUSSION

The only data we have been able to find in the literature concerning the amino acids of gastric juice are those of Martin (*2*, *3*) gained by the original nonspecific Folin method (*4*), and on the gastric juice after histamine. Martin (*3*) found no significant change from normal in the amino-acid nitrogen and a slight increase in the average protein and nonprotein-

nitrogen concentrations in the gastric juice of the patients with gastric ulcers (in the absence of obstruction). On the other hand, in six patients with gastric carcinoma, and no free hydrochloric acid, Martin (*3*) found approximately a fourfold average increase above normal in the nitrogenous constituents, including the amino-acid nitrogen, of the gastric juice; the values for all of the nitrogenous constituents studied were higher in every patient than any of the values found for the "mixtures" of the juices of normal individuals. In benign achlorhydria and in pernicious anemia Martin (*3*) also observed an approximately twofold average increase over the normal in the nitrogenous constituents, including the amino-acid nitrogen of the gastric juice, and raised the question whether the increase in nitrogenous constituents of the gastric juice of these individuals with achlorhydria and of the patients with cancer of the stomach and no free hydrochloric acid could be concerned with altered enzyme activity. Clarke and Rehfuss (*5*) have reported that in some patients with carcinoma of the stomach, and in some patients with gastric ulcer, the gastric juice after an Ewald meal shows an abnormally great increase in protein content, which findings these authors (*5*) attribute to an exudation of protein material from inflammatory, ulcerous, or carcinomatous mucous membranes.

Within limitation, the results of the present study confirm certain of those of Martin (*2*, *3*). The amino-acid values, as gained by the chromatographic methods utilized, and the nonprotein-nitrogen and protein values found in the fasting gastric juice of the patients of this study with gastric or duodenal ulcers (tables 1 and 2) were essentially the same as those found in normal individuals, and the nonprotein nitrogen and protein values found in the gastric juice of fasting normal individuals (*1*) are within the range of the values found by Martin (*2*, *3*) in normal individuals. In all of the six patients of our study with gastric carcinoma very much higher contents of amino acids were present in the gastric juice (tables 2 and 3) than were observed in any of the normal individuals. Although the nonprotein-nitrogen and protein concentrations of the gastric juice of several of these patients with gastric carcinoma (table 3) were well outside the range observed by us in normal individuals, two of the six patients had both nonprotein-nitrogen and protein values within the normal range; in general the juices with the most strikingly increased amino-acid concentrations (table 3) were those in which the nonprotein-nitrogen and protein values were highest.

The greatly increased amino-acid concentrations found in the fasting gastric juice from patients with carcinoma of the stomach may be simply attributable to the presence in the juice of amino acids derived from proteolysis of necrotic or inflammatory products of the cancerous growth. The only other instance in which we found in a juice, uncontaminated with blood or duodenal regurgitations,[6] as high a detectable free amino-acid concentration as those observed in patients with gastric carcinoma,

[6] Hawkins *et al.* (*6*) cite their findings, from chromatographic methods, of minute traces of aspartic acid, glutamic acid, taurine, and of valine in the unhydrolyzed alcohol filtrate of human bile, and of large amounts of taurine with significant amounts of alanine, aspartic acid, glutamic acid, glycine, leucine, and valine after hydrolysis of the alcohol filtrate of bile.

was in a patient who had had an 80 percent gastrectomy 9 days before the juice was aspirated for study; the finding in this instance may also be explicable on the basis of proteolysis of damaged gastric tissue. Certainly we have been able to detect most of the amino acids found in the hydrolysates of tissue proteins in one or another of our chromatograms of the gastric juice of the patients with carcinoma.

The recent two-dimensional paper chromatographic investigations of Roberts *et al.* (*7–9*) of the amino acids of extracts of mouse tumors, "freed of visibly detectable necrotic material," may be pertinent to our findings. In the instance of all of the cancerous tissues examined by these authors, except in the instance of squamous-cell carcinoma, the amino acids were found to be increased above those of the homologous normal tissues; it was further observed that all of the different neoplastic tissues tended to approach the same pattern of free amino acids, whereas each normal tissue had a distribution of free amino acids characteristic of that tissue.[7] That the amino-acid concentration of a gastric juice that bathes cancerous tissue might be increased is perhaps in the light of these findings almost to be expected; it is worthy of note in this respect that Davidson (*11*) and Loofbourow (*12*) have found the amino-nitrogen concentrations of suspension fluids from yeast cells that had been damaged by ultraviolet irradiation to be very much higher than those of suspension fluids from non-irradiated cells.

The chromatographic studies of the free and peptide amino acids in the small number of patients with lesions of the stomach studied in this investigation, do not constitute sufficient material to afford generalizations of the diagnostic significance of the findings. The gastric cancers in the patients of this study were sufficiently advanced to allow diagnosis by clinical and X-ray methods. In one instance (gastric-juice specimen #12, table 2) in which no positive differential diagnosis between gastric ulcer and carcinoma was obtainable by clinical and X-ray studies, the chromatogram resembled those from patients with gastric carcinoma. Whether knowledge of amino-acid concentrations [8] of the gastric juice might prove an adjunctive aid in the detection of early carcinoma of the stomach can only be discovered by further studies. A study of the amino-acid concentrations of the gastric juice by a simple method of chromatography combined with the usual methods of clinical and X-ray diagnosis in a large series of patients with gastric lesions would seem to be indicated at this time. From our findings that gastric-juice specimens contaminated grossly with blood ("coffee-grounds material") or with duodenal regurgitations yielded chromatograms similar to those observed for the juices of patients with cancer of the stomach, it is evident that great care is requisite in selection

[7] Although the findings may not be related pertinently to the following, it is of interest in the light that Greenstein (*10*) has reviewed the evidences that tumor tissues show a distribution of sulfur (cystine-cysteine ratio), and certain vitamin, enzyme, and immunologic reactions with a pattern of resemblance between tumors rather than to the tissue from which the tumor arises.

[8] The finding that the concentrations of alanine, the leucines and valine in the gastric juice of patients with carcinoma were always markedly increased above the normal suggests that quantitative estimation of any one of these constituents might afford a simple method of "screening."

of specimens and in interpretation of the results of studies of the amino-acid concentrations of gastric juice.

SUMMARY AND CONCLUSIONS

The free and peptide amino acid constituents of the gastric juice of fasting patients with gastric and duodenal ulcers and of carcinoma of the stomach have been studied by a method of two-dimensional paper partition chromatography. Measurements of the nonprotein-nitrogen and protein concentrations of the gastric juices of these patients have also been made. The findings have been compared with those for the gastric juice of normal individuals.

The chromatograms of the gastric-juice specimens of six of the seven patients with gastric and duodenal ulcers showed the free and peptide amino acids to be qualitatively and quantitatively the same as found in the gastric juice of normal individuals. In one patient with a gastric ulcer the chromatogram revealed a free amino-acid concentration some three times greater than the average found in the gastric juice of normal individuals.

In all of the six patients with carcinoma of the stomach the chromatograms of the gastric juice revealed very much higher concentrations of free amino acids than were found for normal individuals or for any of the patients with ulcers.

On the chromatograms of the gastric juices of these patients with carcinoma of the stomach not only were the amounts of the amino acids usually detectable on the chromatograms of the juice of normal individuals greatly increased, but more amino acids were usually detectable.

In one elderly patient in whom the differential diagnosis between gastric ulcer and gastric carcinoma could not be established, the chromatogram of the fasting gastric juice revealed a higher concentration of amino acids than found in normal individuals or in any of the patients with gastric or duodenal ulcers.

The chromatographic findings for gastric juices grossly contaminated with duodenal regurgitations and with blood ("coffee-grounds material") are reported and discussed.

In the patients with gastric and duodenal ulcers the nonprotein-nitrogen and protein concentrations of the gastric juice were within the limits found in normal individuals.

In the patients with gastric carcinoma the nonprotein-nitrogen and protein concentrations were usually higher than found in normal individuals. Values within the normal limits of concentration of these constituents were found in some of the patients with gastric carcinoma. However, chromatograms in these patients revealed abnormally high concentrations of amino acids.

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PAPER PARTITION CHROMATOGRAPHY OF FREE AMINO ACIDS AND PEPTIDES OF NORMAL HUMAN SALIVA[1,2]

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INTRODUCTION

It became of interest in conjunction with our recent chromatographic studies of the amino acids of the gastric juice of fasting individuals (*1*, *2*) to have information concerning the amino acid constituents of human saliva, since this latter secretion is present in variable small amounts in the fasting gastric contents. Inasmuch as we were unable to find any publications concerning the identity and concentrations of the free amino acids or peptides of saliva, two-dimensional paper partition chromatographic methods were employed in a study of these constituents in specimens of normal human saliva, and the findings of this investigation are herein reported.

MATERIAL AND METHODS

The material of study consisted of eight specimens of saliva from six normal laboratory workers. The specimens were obtained after the subjects had fasted overnight and during the salivary stimulation of chewing paraffin; the saliva secreted during the first 10 minutes of the chewing period was discarded, and the samples tested were those obtained during the following period of 20 to 40 minutes of continued chewing.

The chromatographic and chemical methods utilized in this investigation were those outlined in detail and employed by the authors in studies of gastric juice and of plasma (*1*, *2*). The chromatograms were run in phenol followed by collidine-lutidine. The free amino acids were chromatographed from amounts of alcohol filtrates and dialysates equivalent to 250 μl. of saliva. The amino acids of the hydrolyzed alcohol filtrates and dialysates were chromatographed from amounts of hydrolysate equivalent to 125 μl. of saliva. The manner and significance of the quantitative estimations of the α-NH_2N concentrations of the individual amino acids from chromatograms as employed here have been discussed elsewhere (*1*). The usage of the terms "detectable," "amino acid," "α-NH_2N," and "peptide" in this text is the same as in a previous report, and explained therein (*1*).

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RESULTS

Spots for alanine, γ-aminobutyric acid, aspartic acid, glutamic acid, and glycine were detectable on the chromatograms of the alcohol filtrates and dialysates of all of the eight saliva specimens studied (table 1). In addition, a weak spot for cysteic acid (from cystine) was usually detectable; taurine was detectable in three of the eight specimens, the leucines in two, and valine, serine, and arginine (the latter two only in the dialysate) were detected in one specimen (#5) (table 1). The chromatograms of saliva were very "weak," both as regards the number of amino acids detectable and the intensities of the spots, in comparison with the chromatograms of the alcohol filtrates or dialysates of plasma run on samples equivalent to the same amount of body fluid (250 μl.) (*1*). The chromatograms run on alcohol filtrates and dialysates of the same saliva specimens were essentially alike except for specimen #5 where the dialysate showed more spots and, in general, stronger spots (table 1).

Quantitative estimations of the individual amino acids showed considerable variations in the different specimens. On the average, γ-aminobutyric acid and glycine together accounted for approximately two-thirds of the total α-NH_2N of the detectable amino acids (table 1). The average estimated total concentration of α-NH_2N of the detectable amino acids was 0.23+ mg. per 100 ml. of saliva with limits of variation from 0.11+ to 0.47+ mg. per 100 ml. (alcohol filtrates, table 1). Previous studies (*1*) indicate that roughly two-thirds of the α-NH_2N of plasma is detectable by the chromatographic method utilized.

Analysis of the chromatograms of the hydrolyzed alcohol filtrates and dialysates of saliva revealed the presence of amino acids in "peptide" (*1*) form (table 1, text-fig. 1). Alanine, aspartic acid, glutamic acid, and glycine were always found in increased amounts after hydrolysis. In addition, proline always became detectable, and occasionally there was a gain of the leucines, lysine, serine, tyrosine, or valine. Quantitative estimations of the α-NH_2N after hydrolysis showed a concentration of total detectable α-NH_2N of 1.9 mg. per 100 ml., as compared with 0.23+ mg. per 100 ml., before hydrolysis (alcohol filtrates and alcohol filtrates hydrolyzed, table 1). The chromatograms of hydrolyzed dialysates and of hydrolyzed alcohol filtrates afforded essentially similar information (table 1). Glycine increased the most markedly of any of the amino acids after hydrolysis and constituted approximately one-half of the total gain. Glutamic acid and proline made up the next most marked increases after hydrolysis; small gains of alanine and aspartic acid were always observed. No consistent increases in γ-aminobutyric acid or taurine were observed after hydrolysis.

The nonprotein-nitrogen concentrations of the specimens of saliva averaged 12 mg. per 100 ml.; the total-protein concentrations averaged 0.16 gm. per 100 ml. (table 1).

TABLE 1.—*Concentrations of free and combined amino acids in normal human saliva, detectable on, and estimated from, chromatograms.*[1] *(Amino acid values are expressed in mg. per 100 ml. of α-amino nitrogen)*

Saliva specimen	#1		#2		#3	#4	#5			#6	
Sample analyzed	Alcohol filtrate	Alcohol filtrate hydrolyzed	Alcohol filtrate	Alcohol filtrate hydrolyzed	Alcohol filtrate	Alcohol filtrate	Alcohol filtrate	Dialysate	Dialysate hydrolyzed	Alcohol filtrate	Dialysate
Alanine	0. 03	0. 07	0. 03	0. 12	0. 01	0. 01	0. 03	0. 05	0. 10	0. 02	0. 02
γ-aminobutyric acid	0. 05	0. 07	0. 04	0. 18	0. 03	0. 02	0. 02	0. 06	0. 06	0. 08	0. 04
Arginine	.	.	.	.	.	.	.	0. 04	0. 10	.	.
Aspartic acid	0. 01	0. 08	0. 02	0. 12	0. 01	0. 02	0. 02	0. 03	0. 11	0. 02	0. 02
Cysteic acid (from cystine)	.	?	W	W	?	W	W	W	W	W	W
Glutamic acid	0. 01	0. 24	0. 02	0. 36	0. 01	0. 01	0. 02	0. 01	0. 12	0. 02	0. 03
Glycine	0. 07	0. 54	0. 18	0. 59	0. 07	0. 03	0. 06	0. 10	0. 37	0. 03	0. 04
Leucines	.	.	0. 04	0. 10	.	.	0. 01	0. 03	0. 04	.	.
Lysine	.	.	.	0. 15	.	.	.	.	.	.	.
Proline	.	W	.	S	.	.	.	W	W	.	.
Serine	.	0. 24	.	0. 22	.	.	.	0. 02	0. 06	.	.
Taurine	.	.	0. 04	0. 15	.	0. 02	0. 01	0. 03	0. 02	.	.
Tyrosine	.	.	.	0. 16	.	.	.	.	.	.	.
Valine	.	.	.	0. 02	.	.	0. 01	0. 03	0. 02	.	.
Total	0. 17+	1. 2+	0. 37+	2. 2+	0. 13+	0. 11+	0. 18+	0. 40+	1. 0+	0. 17+	0. 15+
NPN mg. per 100 ml	10	---	13	---	10	13	10	10	---	---	---
Protein gm. per 100 ml [2]	0. 19	---	0. 23	---	0. 07	0. 22	0. 16	---	---	---	---

See footnotes on page 694.

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TABLE 1.—*Concentrations of free and combined amino acids in normal human saliva, detectable on, and estimated from, chromatograms.*[1] (*Amino acid values are expressed in mg. per 100 ml. of α-amino nitrogen*)—Continued

Saliva specimen	#7				#8			Average value and limits	
Sample analyzed	Alcohol filtrate	Dialysate	Alcohol filtrate hydrolyzed	Dialysate hydrolyzed	Alcohol filtrate	Dialysate	Alcohol filtrate hydrolyzed	Alcohol filtrate	Alcohol filtrate hydrolyzed
Alanine	0. 03	0. 03	0. 18	0. 17	0. 02	0. 02	0. 07	0. 02(0. 01–0. 03)	0. 11(0. 07–0. 18)
γ-aminobutyric acid	0. 07	0. 07	0. 09	0. 10	0. 24	0. 16	0. 27	0. 07(0. 02–0. 24)	0. 15(0. 07–0. 27)
Arginine	.	.	.	.	.	.	.	.	.
Aspartic acid	0. 01	0. 02	0. 11	0. 17	0. 01	0. 01	0. 08	0. 02(0. 01–0. 02)	0. 13(0. 08–0. 23)
Cysteic acid (from cystine)	W	W	W	W	W	W	W	W (N.D.–W)	W (N.D.–W)
Glutamic acid	0. 03	0. 04	0. 33	0. 33	0. 01	0. 02	0. 23	0. 02(0. 01–0. 03)	0. 29(0. 23–0. 36)
Glycine	0. 06	0. 12	*1. 47*	*1. 04*	0. 19	0. 12	*0. 80*	0. 09(0. 03–0. 19)	*0. 85*(0. 54–*1. 47*)
Leucines	.	.	0. 12	0. 12	.	.	0. 08	0. 02(N.D.–0. 04)	0. 10(N.D.–0. 12)
Lysine	.	.	0. 29	0. 16	.	.	0. 03	.	0. 16(N.D.–0. 29)
Proline	.	.	S	S	.	.	S	.	M (W–S)
Serine	.	.	.	.	.	.	.	.	0. 23(N.D.–0. 24)
Taurine	.	.	.	.	.	.	.	0. 02(N.D.–0. 04)	(N.D.–0. 15)
Tyrosine	.	.	0. 02	.	.	.	.	.	0. 09(N.D.–0. 16)
Valine	.	.	0. 04	0. 06	.	.	0. 04	(N.D.–0. 01)	0. 03(N.D.–0. 04)
Total	0. 20+	0. 28+	*2. 7*	*2. 2*	0. 47+	0. 33+	*1. 6*	0. 23+(0. 11+–0. 47+)	*1. 9*(1. 2+–2. 7)
NPN mg. per 100 ml	17	17	------	------	13	------	------	12 (10–17)	------
Protein gm. per 100 ml [2]	0. 18	------	------	------	0. 10	------	------	0. 16(0. 07–0. 23)	------

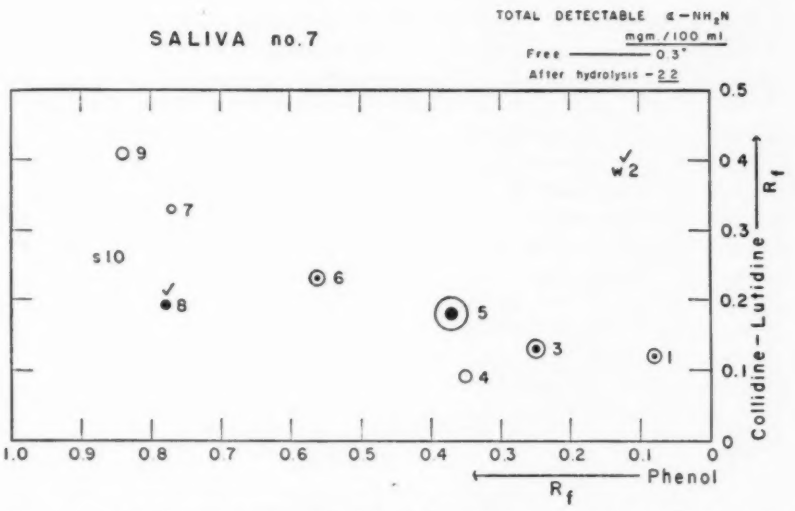
[1] See Methods. An italicized value signifies that the "factor" for the amount of saliva chromatographed was greater than 150. The dots indicate that the amino acid was not detectable in the amount of saliva chromatographed.

The "?" marks for cysteic acid (from cystine) signify that no separate chromatograms were run with hydrogen peroxide.

"W", "M", and "S" signify weak, medium, and strong color intensities of spots, respectively.

The averages on the table have been recorded in the following manner: With two or more detectable spots for a given amino acid in a series (as for the alcohol filtrates) the average of the two or more estimated concentrations of α-amino nitrogen has been recorded and the limits of the findings expressed as "(x–x)" or as "(N.D.–x)", "N.D." signifying that the amino acid was not always detectable. When a spot for an amino acid has occurred only once in a series, no average is recorded, but the limits of "(N.D.–x)" are shown. The averages of the totals were calculated from the total of each individual specimen analyzed.

[2] N. B. The protein values were measured on saliva filtered through gauze and S. & S. #604 paper.



TEXT-FIGURE 1.—Graphic representation of the chromatograms of the amino acids before and after hydrolysis of a dialysate of a typical specimen of saliva.

The areas of the spots are in direct proportion to the concentrations of the α-NH_2N as estimated from the "factors" gained from the actual chromatograms. Because the intensity of the spots is not depicted here, the sizes are not in the same relation as on the original chromatograms (*1*).

The solid spots represent the concentrations of the α-NH_2N of the free amino acids, the circles represent the concentrations of the α-NH_2N of the amino acids after acid hydrolysis of the dialysate.

"W" and "S" signify weak and strong spots, respectively, for cysteic acid and proline; the latter being detectable only after hydrolysis.

A "√" signifies that the amount was the same before and after hydrolysis.

The numbers on the figure identify the amino acids as follows: 1. aspartic acid; 2. cysteic acid; 3. glutamic acid; 4. lysine; 5. glycine; 6. alanine; 7. valine; 8. γ-aminobutyric acid; 9. leucines; 10. proline.

DISCUSSION

In the course of our chromatographic studies of the amino acids of human gastric juice we obtained a specimen of gastric "contents" from a healthy young nurse who had great and prolonged difficulty in swallowing the Levin aspirating tube. Chromatographic analysis of this specimen of gastric "contents" revealed the concentration of total free amino acids to be much less than previously encountered by us on analyses of normal gastric juice, and the "pattern" of the amino acids on the chromatogram to be quite different from that recognized for gastric juice! It was suspected, naturally, that the gastric "contents" finally obtained from this subject was to a large extent comprised of saliva;[4] this suspicion prompted us to make the studies herein reported.

The variations found in the chromatograms of the amino acids of the different specimens analyzed was expectable, inasmuch as saliva is com-

[4] Comparison of the findings from the chromatogram of the gastric "contents" of this subject with those later obtained from analyses of saliva specimens revealed this to be true.

posed of a mixture of the characteristic secretions of several glands. It has been found by others that the composition of saliva varies, as does that of gastric juice, with different secretory stimuli (*3*). Some of the variations encountered in the data (table 1), may be due to inaccuracies inherent in the chromatographic method of estimation (*1*).

The chromatograms of the free amino acids of normal saliva (table 1, text-fig. 1) differ markedly from those of both gastric juice and of plasma. In saliva some two-thirds of the total detectable free α-NH_2N is comprised of glycine plus γ-aminobutyric acid; in contrast, in gastric juice some two-thirds of this total is comprised of the leucines plus valine and γ-aminobutyric acid, and in plasma approximately one-half is comprised of glutamine plus the leucines. Further, the total amount of detectable free amino acids in normal human saliva is small in comparison with that of gastric juice and plasma; from our chromatographic studies it is estimated that, on the average, the total detectable free α-NH_2N of saliva is only approximately 25 percent and 10 percent of that of gastric juice and plasma, respectively.

The concentration of the detectable amino acids obtained from hydrolysis of the peptides of saliva greatly exceeds (by some eight times) that of the free amino acids (table 1, text-fig. 1). Similarly the peptide α-NH_2N of gastric juice exceeds that of the free α-NH_2N very greatly (*1*, *2*). The concentration of α-NH_2N from the peptides of saliva would appear to be some one-fifth of that of gastric juice from normal fasting individuals. The findings of "high" peptide concentrations in saliva and gastric juice are to be contrasted with the relatively low (especially in proportion to the free amino acids) peptide concentration of plasma from normal fasting individuals (*4*).

The nature of the peptides present in saliva and the mechanism of their secretion (or manner of gaining presence) are beyond the scope of the studies of this report. No spots that could be attributed to peptides, *per se*, were seen on the chromatograms.

The values for nonprotein-nitrogen and protein concentrations of saliva found in this study (table 1) agree with the findings of others (*3*). The nonprotein nitrogen of saliva is reported to be comprised to a large extent of urea plus ammonia nitrogen, and the protein to be comprised largely of mucin together with small amounts of albumin and globulin (*3*).

SUMMARY AND CONCLUSIONS

Qualitative information and quantitative estimations of the free and "peptide" amino acids of eight specimens of normal human saliva were gained through studies employing a two-dimensional paper chromatographic technic.

Alanine, γ-aminobutyric acid, aspartic acid, glutamic acid, and glycine were detectable on the chromatograms of all the specimens of saliva studied. Certain other amino acids were sometimes detectable. The chromatograms of the free amino acids of saliva differ greatly from those for plasma and gastric juice.

The average total detectable free α-NH_2N concentration of saliva was estimated to be 0.23 + mg. per 100 ml. γ-aminobutyric acid plus glycine comprise some two-thirds of the total estimated concentration of detectable α-NH_2N of the saliva.

The concentration of the α-NH_2N from "peptides" in saliva exceeds the concentration of free α-NH_2N by approximately eight times.

The average total detectable free α-NH_2N concentration of saliva is approximately only 25 percent and 10 percent of that of gastric juice and plasma, respectively. The concentration of α-NH_2N from peptides in saliva is only approximately 20 percent of that in gastric juice and somewhat greater than that in plasma.

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